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The key objective of these studies is to determine the neurotoxic risks of combining acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor blockers. In this year of study we found: 1) The histopathological effect of the non-competitive NMDA antagonist MK-801 is ameliorated by co-exposure to the acetylcholinesterase inhibitor, pyridostigmine bromide in rats; however neurotoxicity is exacerbated by co exposure to the AChEI, physostigmine. 2) The NMDA receptor antagonists: dextromethorphan and felbamate do not induce neurotoxicity in exposed animals, nor does co-exposure of these compounds to pyridostigmine bromide induce detectable neurotoxicity. 3) The NMDA receptor antagonist, memantine induces a neurotoxic response visualized by positive Fluoro-Jade-B stain in mature female rats; this effect is exacerbated by pyridostigmine bromide. Several animals died with these drug combinations, suggesting this is a toxic combination. 4) The resultant neuropathology in MK-801 and memantine exposed animals is in good agreement with the behavioral deficits exhibited by animals exposed to these compounds. 5) Combined exposure of memantine and PB had a greater effect on IPSPs than did memantine or PB alone. The major finding of this year is that memantine, in combination with an anticholinesterase, can be quite neurotoxic and perhaps lethal in rats. Human use of this drug combination should be carefully reviewed and further research is necessary.

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Introduction

The key objective of these studies are to determine the neurotoxic risks of combining acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor blockers. We chose to investigate the neurotoxic effect of combined exposures the AChEI agents, physostigmine and pyridostigmine bromide with different NMDA receptor antagonists using behavioral and histopathological assessments of toxicity in mature female rats.

Body

NMDA Antagonist-Induced Model of Neurotoxicity

In our original proposal for this project for the Department of Defense, we chose to use an animal model of neurotoxicity that, at the time, was relatively new - and some of the model's applications were still untested. However, in the intervening years, this model has proven to be far more interesting even we expected, and the results from the many studies focused on this neurotoxicity may have wide-ranging significance for both military and civilian medicine.

Specifically, we chose to use a model of neurotoxicity that employs NMDA receptor/channel antagonists ("NMDA antagonists") to produce neuronal damage - and to assess the interaction of NMDA antagonists with chemical agents that resemble nerve agents such as sarin and soman (i.e., the anticholinesterase inhibitors, or AChEI). We chose this approach based on the hypothesized risk of co-exposure to these two agents, and the potential for a synergistic interaction that could put a co-exposed individual at far greater risk of permanent brain damage than would be expected, based on the effect of either agent alone.

Furthermore, one hallmark of NMDA antagonist-induced neurotoxicity is the region-specific damage it produces in the brain. However, damage to these vulnerable areas may produce significant effects on human behavior and functioning, but because those areas are involved in complex emotional processing and higher executive functions,

evidence of the damage might be difficult to detect outwardly - even though the actual outcome is a profoundly impaired individual. Some of the brain regions most vulnerable to this neurotoxicity were already known to be important for military issues (e.g., the amygdala and the processing of fear and threat), whereas other areas were less well understood in humans (e.g., the posterior cingulate cortex and retrosplenial gyrus). However, recent studies have shown light on the function of these areas - and the possible repercussions neurotoxic damage to these brain areas may have special significance in military settings, as will be discussed below.

Furthermore, this model has particular significance to medical treatment of individuals in active military duty as well as veterans, for new drugs based on these mechanisms are being developed to treat Parkinson's Disease (Parsons et al., 1999) and pain (Fisher et al., 2000; Hewitt, 2000; Schmid et al.,1999), as well as many other neurological conditions that are significant for good medical care of U.S. veterans and their families. Specific to military conflict, is the potential usefulness of NMDA antagonists to treat life-threatening seizures that arise after exposure to sarin, soman or related nerve agents. It is, perhaps, this latter potential use that may be most relevant to the current international state of affairs, in which the risk of exposure to nerve agents seems far more likely than ever before.

In summary, the questions we have addressed in this proposal appear to be even more significant than ever, given the wider range of therapeutic drugs with potential NMDA antagonist activity, the significance of the areas of the brain affected and, unfortunately, the ever-greater risk of nerve gas exposure.

NMDA Antagonist Neurotoxicity

In our previous Annual Reports (2000 and 2001), we went into depth regarding this new model of neurotoxicity produced by NMDA antagonists, as well as the mechanistic basis of the possible interaction between NMDA antagonists and AChEIs. This included the background studies (Olney et al., 1989 & 1991), and the subsequent studies done based on those initial findings. Rather than re-state this information at great

length, we instead will briefly summarize these background studies, and then later go into more detail into the more recent studies and the relevance of those studies to our results.

History of the Model

When endogenous excitatory amino acids (EAAs) were first discovered to produce excitotoxicity in the CNS, the clinical repercussions seemed almost unlimited. Almost immediately it was recognized that if endogenous EAA's damage the brain, then the antagonists of these receptors might hold incredible promise as neuroprotective drugs (Rothman & Olney, 1995). Special attention was focused on antagonists of the N-methyl-D-aspartate receptor/channel complex (NMDA antagonists), given their effectiveness in certain *in vitro* and *in vivo* animal models.

However, in the process of evaluating NMDA antagonists, they were found to have unusual behavioral side effects in some of the animal models. Even more troubling were the reports of CNS problems in human clinical trials. For example, the drugs caused severe psychotic reactions in a clinical trial using NMDA antagonists in patients with epilepsy (Sveinbjornsdottir et al., 1993), NMDA antagonists caused schizophrenia-like symptoms in normal volunteers (Javitt et al., 1991; Krystal et al., 1994), and chronic use of PCP in humans produces persistent psychotic symptoms that has actually suggested its usefulness as a chemical model of schizophrenia (Jentsch & Roth 1999). However, it was not clear if these problems were also associated with neurotoxicity, or were simply reversible behavioral effects.

Evidence suggestive of neurotoxicity in animals was provided by an important set of studies by Olney and colleagues (Olney et al., 1989 & 1991). These investigators found that several NMDA antagonists produced signs of neuronal injury (vacuolization in H & E stained tissue); later studies provided more evidence of possible cell death (e.g., positive silver-staining in neurons (Corso et al., 1997)). The effects were dose-dependent and region-specific, with the limbic cortex being among the most vulnerable anatomical areas.

Regions of Vulnerability

Within the limbic cortex, the most sensitive areas are the posterior cingulate cortex and the retrosplenial cortex (PCC/RSC). Higher doses produced more widespread damage, including the anterior cingulate, the amygdala, and other cortical areas. Recent studies also have reported that the NMDA antagonist phencyclidine (PCP) damages a subpopulation of neurons in the striatum (Mitchell et al. 1998), and also that the prototypical NMDA antagonist MK-801 damages neurons of the anterior thalamus (Tomitaka, et al., 2000).

These findings have subsequently been extended to a wide range of NMDA antagonists (Olney 1994), including memantine (Tomitaka,et al., 1996) and ketamine (Ellison 1995). In addition to neuropathological changes in tissue stained with H & E, signs of neuronal injury have been detected with a wide range of techniques, including silver stains (Corso et al., 1997), TUNEL staining for apoptosis (Mitchell et al., 1998), and heat shock protein (HSP 70) elevation (Sharp et a.l, 1993 & 1994; Tomitaka et al., 1996). This effect has also been seen in other animal models, such as mice (Woznick et al., 1996). It is not yet known if neuronal damage occurs in humans, but NMDA antagonists produced enhanced blood flow in areas of the human brain that may have parallels with the animal studies (Krysta et al., 1994).

Depending on the anatomical regions studied, certain cell types showed NMDA antagonist-induced damage. In limbic cortical areas, cells in the deep cortical layers (III & IV) showed rapidly developing (though reversible) vacuolization with moderate doses (Olney et al., 1989). Higher doses produced evidence of cell death, as measured by several histological methods (Olney et al., 1991; Corso et al., 1997). In the striatum, PCP produced damage in striatopallidal neurons (Mitchell et al., 1998).

The Effect of Age and Gender

NMDA antagonist-induced neurotoxicity has also been found to be age- and gender-dependent: older animals are much more vulnerable than younger animals(Olney et al., 1991; Auer 1996) and females are more vulnerable than males (Hönack & Löscher

1993; Auer 1996). The age effect is in parallel with the side effect profile in humans given the anesthetic agent ketamine, the use of which the use is limited to children because of the unacceptable side effect profile in adults (Reich & Silvay 1989).

Proposed Pharmacological Mechanisms

Many neurotransmitter systems modulate NMDA antagonist-induced neurotoxicity, because toxicity is exacerbated or blocked by drugs acting upon different types of receptors. For example, pilocarpine, a muscarinic cholinergic agonist, greatly exacerbated the neurotoxicity of the NMDA antagonist PCP (Corso et al., 1997) and this toxicity could be reduced or blocked by muscarinic cholinergic antagonists such as atropine (Olney et al., 1991). Other protective agents included GABAergic drugs such as benzodiazepines (Olney et al., 1991), antipsychotics such as haloperidol and olanzapine (Sharp, et al., 1993; Farber, et al., 1996), and selective serotonin reuptake inhibitors (SSRIs) (Tomitaka et al. 2000). In addition, not all NMDA antagonists produce neuronal damage. The glycine site antagonists, as a group, appear to be better tolerated (Hawkinson et al., 1997; Hargreaves et al., 1993; Koek & Colpaert 1990).

This is an important model of neurotoxicity, given the potential usefulness of the NMDA antagonists for a range of conditions, including chronic pain (Schmid & Sandler 1999), soman-induced seizures (Filliat et al., 1999), schizophrenia (Greene 2001; Sharp et al., 2001; Tsai & Coyle 2002) and Parkinson's disease (Mitchell & Carroll 1997). This includes drugs for which NMDA receptor antagonist activity could be one of several co-existing therapeutic mechanisms, one or more of which may contribute to the clinical effectiveness of that drug (e.g. the anti-Parkinson's agent memantine (Tomitaka et. al, 1996) or the anticonvulsant felbamate (Rho et al., 1994)).

NMDA antagonists and AChEI Nerve Agents

The use of anticonvulsants may have special relevance in military settings, for it was found that soman-exposed rats that had exhibited soman-induced convulsions were likely to have hippocampal damage (in area CA1) and memory impairment, whereas rats

exposed to an identical dose of soman, but in which convulsions did not occur, were spared (Filliat et al., 1999). Accordingly, proper treatment of post-nerve gas exposure seizures may be critical to preventing neurotoxicity (McDonough & Shih 1995). Also relevant to this topic is the fact that the NMDA antagonist MK-801 provided protection against soman-induced seizures in vivo (Braitman & Sparenborg,1989), and soman-induced status epilepticus was reduced by the NMDA antagonist N-[1-(-2-thienyl) cyclohexyl]-piperidine (TCP) (Carpentier et al. 1994) (although these agents were not without problems (Filliat et al., 1999).

The interaction between NMDA antagonists and the cholinergic system may also be important for blocking neurotoxicity due to agents like soman, because drugs with mixed actions as both anticholinergics and NMDA antagonists may hold special promise for use against nerve agent-induced seizures. This may be due, in part, to the stages through which nerve agent-induced seizures progress. Specifically, after exposure to a nerve agent, there is a sequence of sensitivity to anticonvulsant drugs that may be clinically important. First, there is a stage of sensitivity to anticholinergic drugs, followed by a transition phase, and then a late phase during which anticholinergic agents are no longer effective (whereas, benzodiazepines and NMDA antagonists are effective if they are co-administered with anticholinergics) (McDonough & Shih 1997)).

These findings have important clinical implications, for effective treatment during the early phase of seizures could prevent neuropathology, but if treatment was delayed until the late phase was reached, neuropathology occurred. It is important to determine if there are safe and effective ways to lessen this toxicity when given acutely, and also if these drugs are going to be given chronically (as in treating chronic pain, schizophrenia, epilepsy, and Parkinson's disease). It is possible that certain of the better-tolerated agents might be highly useful in a military setting, as long as their toxicity can be blocked with therapeutic or dietary preventive measures.

Proposed Mechanisms of Neurotoxicity of NMDA Antagonists

The exact mechanism by which NMDA antagonists produce neurotoxicity is still under investigation. Early on, Olney and colleagues proposed that disinhibition was a

possibility, but studies directly aimed at this mechanism were yet to be done. However, in a recent set of in vivo studies, Sharp and colleagues showed that there is a pivotal role for the anterior thalamus (AT) and the NRT in NMDA antagonist neurotoxicity: they found that the toxicity of systemic administration of MK801 could be blocked by direct bilateral injection into the anterior thalamus of GABAa receptor agonists (Sharp et al., 2001). In contrast, they found that direct injection of MK801 into the RSC did not cause damage. The authors propose that NMDA antagonists cause disinhibition in the thalamus, resulting in enhanced output from excitatory AT cells that then contribute to excitotoxicity in the PCC/RSC and other vulnerable areas.

However, they note that thalamic disinhibition cannot be the whole story, because the AT sends synaptic contacts to many cortical areas of the brain, but only certain areas are damaged by NMDA antagonists (Sharp et al., 2001). An answer to this puzzle may have been provided in our recent report, in which MK801 produced disinhibition in cortical slices, but the C/RSC was significantly more sensitive to the disinhibitory effects of MK801 than the parietal cortex (Li et al., 2002). Since the RSC also is more sensitive to NMDA antagonist toxicity than the parietal cortex, this disinhibitory effect of MK801 is in parallel with the anatomical vulnerability of these areas. Taken together, the results from these two groups may explain the region-specific damage seen with NMDA antagonists.

Functional Effects of NMDA Antagonist-Induced Neurotoxicity

Based on the regions damaged, the functional effects of NMDA antagonist-induced damage may be obvious, or it may be subtle, but still very debilitating. The damage produced by PCP in the striatum may have relevance to serious movement disorders, including Huntington's Disease (Mitchell et al., 1998). Recent animal studies report that RSC damage produced deficits in spatial memory in rats (Vann & Aggleton 2002).

As a whole, the cingulate gyrus has been the focus of a number of studies, but only recently have the posterior cingulate or retrosplenial cortex been the focus of functional studies. Before these studies, there was not a clear understanding of what role

the more posterior regions play in human behavior. However, that has changed recently, and some of these studies have been spurred on by Olney's findings of region-specific NMDA antagonist neurotoxicity in animals. This recent burst of activity has yielded a number of studies from which is emerging a very interesting picture with respect to functional effects of damage in the anterior cingulate cortex (ACC), the PCC/RSC, and other limbic and cortical areas vulnerable to NMDA antagonists.

In humans, the ACC, PCC and RSC appear to be involved in a wide range of cognitive, emotional, and behavioral processes. These include the involvement of the ACC in pain perception (Harold et al., 1999), the involvement of the RSC or PCC in emotional processing or movement (Maddock 1999), the role for cingulate activity in executive functions and evaluational processes (Vogt et al., 1992), the role of the ACC in psychiatric disorders (e.g., depression and obsessive-compulsive disorder), and neurological deficits in attention and language processing in Parkinson's Disease (Grossman et al., 1992), and the PCC/RSC in circuitry for fear and anxiety (Charney and Deutch 1996).

Pain Perception

In military combat and recovery settings, as well as civilian clinical settings, one very difficult medical issue involves the treatment of intractable pain associate with amputations (phantom pain) or neuropathic pain from damaged nerves. The role of the cingulate (and associated areas) has long been the focus of pain control in humans, especially with respect to neurosurgical treatments (e.g., Foltz & White 1962 and references therein; Vogt et al., 1992). Given the continued difficulty in treating certain types of chronic intractable pain with traditional analgesics, this focus has only expanded.

Recent reports have included more direct studies in awake, behaving individuals during surgery. For example, Hutchinson et al., (1999) measured neuronal activity *in situ* in humans during neurosurgical procedures to remove the anterior cingulate cortex for treatment of chronic depression or obsessive-compulsive disorder. Before the cingulotomy took place, microelectrodes were implanted in the ACC to measure responses of individual neurons to painful stimuli in the periphery. (The patients were

awake and no analgesics were present). Neurons in the ACC responded to a variety of noxious stimuli (painful heat cold, or pinch) but not to innocuous stimuli. The role of the ACC appeared to be in pain perception, because direct stimulation of the neurons did not produce a sensation of pain. Thus, damage to this area could be expected to produce impaired pain perception which, if causes an increased perception of pain, might not be expected to respond to typical analgesics.

The PCC and Moral Reasoning

A very interesting study in humans was recently published in which the posterior cingulate cortex (PCC) was found to be involved in reason and emotional judgment (Greene et al., 2001). In fact, the PCC appeared to show the most robust effect, for it was the brain area that was significantly activated across 2 different experimental tests.

Specifically, the investigators used fMRI to explore the areas of the brain involved in making moral judgments - with a special focus on the difference between moral judgments that had serious outcomes and involved careful emotional processing. The authors presented the normal volunteer subjects with a range of moral dilemmas, about which the subjects had to chose one of two responses: appropriate or inappropriate. The dilemmas included those types of decisions that are extremely painful to make, even in theory (e.g., having to make decisions involving serious issues and direct harm to one or more individuals). By comparison, other dilemmas were simple and involved little emotional processing or moral judgment (e.g., whether to take the train or the bus, if time is limited).

One example the authors used was the ethical dilemma called "the trolley dilemma":

> "A runaway trolley is headed for five people who will be killed if it proceeds on its present course. The only way to save them is to hit a switch that will turn the trolley onto an alternate set of tracks where it will kill one person instead of five. Ought you turn the trolley to save five people at the expense of one?"

As the authors discuss, most people say yes, it would be appropriate to turn the trolley, even if it meant that the one person would die.

In contrast, is the "footbridge dilemma":

> "As before, a trolley threatens to kill five people. You are standing next to a large stranger on a footbridge that spans the tracks, in between the oncoming trolley and the five people. In this scenario, the only way to save the five people is to push this stranger off the bridge, onto the tracks below. He will die if you do this, but his body will stop the trolley from reaching the others. Ought you save the five others by pushing this stranger to his death?"
>
> To this dilemma, most people say, "no, it is not appropriate to push the stranger off of the footbridge".

Why does it seem appropriate to sacrifice a stranger in one situation but not the others? Simple cognitive reasoning may result in both options seeming to be equal. Yet, why do most people seem to agree about the two different answers? The authors suggest that there is a personal involvement in the footbridge scenario that is less powerful in the trolley switch scenario. This personal involvement also employs more emotional moral judgment. And, in accord with this proposal is the degree to which areas of the brain involved in special emotional processing "light up". Interestingly, the posterior cingulate was the only area out of the four areas studied which "lit up" in the two different versions of the tests administered to the subjects.

Accordingly, the PCC may be critical in helping humans make "appropriate" moral judgments in highly emotional settings - especially when simple cognitive reasoning might suggest that: "one person is equal to the other - so both choices are appropriate". Thus, it is possible that neurotoxic damage in the PCC may have profound effects on sound moral reasoning (and perhaps even without producing any dramatic behavioral deficits with regard to more mundane tasks).

In the civilian world, most individuals seldom have to make life-or-death moral decisions. This is not the case in the military settings, especially during periods of conflict, when individuals may need to make serious moral decisions - and do it often. Subtle changes in moral judgment could have serious outcomes for all individuals involved - to those to whom the decisions has a direct impact - perhaps even life-threatening impact, as well as to the decision-maker (who will have to suffer from the painful memories of the bad decisions for a lifetime). In addition, probably few things are more painful than having moral judgment questioned - especially later, when there is nothing to do but re-live the horror. And, by its very nature, such after-the-fact scrutiny can occur decades later, but the delay in no way attenuates the devastating effects, even to honorable individuals who made a decision that, at the time, most others would have though was appropriate - or, at least, understandable.

It is very important to point out that, at present, any links between PCC damage, loss of moral judgment, and NMDA antagonist neurotoxicity (and AChEI interactions) are highly speculative. Obviously, these are not the kinds of questions that can be answered with rat models. Even in humans, the only way certain questions might be addressed is to study humans exposed to these agents either accidentally (e.g., drug overdoses or in toxic spills) or intentionally (e.g., in drug abuses who use PCP, or individuals exposed to AChEI nerve agents and NMDA antagonists in terrorist attacks). Nevertheless, the potential for such an outcome should be taken seriously - especially in a population in which serious moral judgments can be required - and repeatedly - during armed conflict.

Background Conclusions

Finally, as we are completing our DOD sponsored research with this model, we are even more convinced that this is an important area in military neurotoxicology. This is especially true, given the subtle, but troubling, possible signs of neurotoxicity in the Veterans of the Persian Gulf War (Golomb 1999; Haley et al., 1997; Riddle et al., 2000; Sartin 2000).

Materials and Methods

Previous studies have shown that female rats are more sensitive to NMDA receptor induced neuronal toxicity (Honack and Loscher 1993; Fix et al., 1995; Guo-Ross et al., 2002), thus, only adult female Sprague-Dawley retired breeder rats were used in this study. Animals had an average weight of 402.6g (+/-5.09 SEM). Rats were ordered from Charles River (Raleigh, NC), housed in groups of two-three animals in clear polycarbonate cages (27.9 X 27.9X 17.8 cm^3) on a 12 hr light/dark cycle at the Durham Veterans Affairs Medical Center Vivarium. All efforts were made to minimize animal suffering and to reduce the number of animals used. Three days after arrival, the animals from each age group were individually weighed and randomly assigned to control or treatment groups. Rats were given drugs or vehicle using intra-peritoneal (i.p.) or subcutaneous (s.c.) injections in a volume of 1.0mg/ml. Drugs were prepared fresh on day of injection in sterile saline (0.9%) or other solvent (eg., DMSO to dissolve the dextromethorphan). Rats were first injected with the NMDA receptor antagonist of interest (MK-801, memantine, felbamate and dextromethorphan), followed within 15 minutes by injection with an AChEI agent ((-) -physostigmine and PB).

Drugs

All drugs were purchased from Sigma, St. Louis, MO. The following drug doses were given: (+)MK-801: 0.3mg/kg, (-)-physostigmine sulfate (physostigmine): 0.1, 0.3 and 1.0mg/kg, pyridostigmine bromide (PB): 0.1, 0.3 and 1.0mg/kg, memantine: 25, 50 and 75mg/kg, felbamate: 100, 300 and 400mg/kg, dextromethorphan: 10, 20 and 50 mg/kg, atropine sulfate 25mg/kg, and (-)- nicotine hydrogen tatrate 0.4mg/kg.

Histology

After injections, rats were placed in a clear polycarbonate cage and observed for drug-induced behavior changes using a functional observation battery (FOB, described below) at one hour, four hours, twenty-four hours and forty-eight hours.

Three days after drug injections the rats were weighed, deeply anesthetized by Halothane inhalation, and transcardially perfused with 150-200ml of heparinized 0.9% saline, followed by 350-400ml 4% buffered paraformaldehyde. The brains were removed and post-fixed in 4% buffered paraformaldehyde overnight, then transferred to 0.1M phosphate buffer (PB) for 24 hours; finally, the brains were soaked in 30% sucrose in 0.1M PB until they sank. Each brain was cut into 40-micron thick sections using a cryostat in a rostral-to-caudel direction. Twelve serial sections (approximately every sixth section beginning at -0.3mm from bregma) were mounted directly to 0.5% gelatin-coated slides. The remaining sections were transferred to 0.1M PB and stored at 4°C for future use. The sections were stained with the fluorochrome: Fluorojade-B (FJ-B) (Histo-Chem Inc., Jefferson, AR) using methods previously described (Schmued et al., 1997; Schmued and Hopkins 2000). Briefly, the sections mounted from the cryostat were allowed to dry on slides overnight at room temperature, then were sequentially processed in the following solutions: 100% ethanol (3 min.), 70% ethanol (1 min.), deionized water (1 min.), 0.06% potassium permanganate (7 min., slow shaking), deionized water (1 min.), 0.004% FJ-B (in 0.1% acetic acid) (30 min. slow shaking) in the dark, then deionized water (three times, one minute each). Slides were then sequentially dehydrated in 70%, 90% and 100% ethanol (5 min. each solution, two times in 100% ethanol). Slides were finally placed in xylene (5 min., two times), then cover-slipped with distyrene plastcizer xylene (Electron Microscopy Sciences, Washington, PA). The stained sections were examined with a Zeiss Axiophot microscope using epifluorescence and a FITC filter. Neurotoxicity was assessed by counting the number of positively stained neurons in the cingulate cortex (CC)- retrosplenial cortex (RSC) region in each section. Digital photographs were made using a 10X dry lens and a 25X water immersion lens, and a Nikon Cool-Pix 990 camera. Photographs were downloaded into Adobe photoshop as JPEG files using Nikon-View software.

Functional observation battery (FOB)

The design and measurements taken by the functional observation battery we used were based on observation batteries designed by others- to specifically distinguish the

different effects of neurotoxicants by observing various neurobehavioral changes in rodents (Irwin 1968; Moser 1990; 1996; Gad 1982; Tilson 1992; United States E.P.A., 1998). The behavioral tests were administered to each animal prior to drug exposure (pre-drug), and additionally at one, four, twenty-four and forty-eight hours post-drug exposure by an observer that was blind to the treatment of each animal. The observer began the test panel (Table 1) by first observing each rat in its home-cage environment, then by observing each animal after it was removed to another cage for observation and manipulation (the arena).

Each home-cage observation was performed in the same sequence, beginning with an unobtrusive assessment of the animal's body posture, bizarre behavior, tremors, twitches, tonic or clonic convulsions, exophthalmos, and eye-crustiness. Body posture was rated on a scale of 1-10, with normal alert behavior scored at 1, and 10 describing a rat that is lying completely flat. Any bizarre behavior observed was given a score between 1-3 based on the degree of severity with a three as most severe. A brief description of the bizarre behavior was also noted. Both head-weaving and aimless wandering were typical bizarre behaviors that were observed. Tremors and twitches were also both scored (1-5); tremors were further described as exertion, head or tail tremors. Severe tremors or twitching rated higher scores. Convulsions were rated as either clonic or tonic and scored descriptively within those categories (between 1-3 for clonic convulsions and 1-5 for tonic convulsions). For example, clonic chewing behavior (scored as a one) is described as less severe than repetitive whole-body clonic tremors (scored as a three), and tonic opisthotonus (scored as a two) was described as less severe than popcorn convulsions (scored as a four). Finally, the presence of either exophthalmos or eye-crustiness was scored with a one (for present) or a zero (for absent).

After completion of the home-cage observations, the observer removed each rat to weigh (once each day of the study), then to a fresh cage to record the following: the animals state of arousal when removed from the home-cage (scored between 1-6, with a limp rat showing an absence of normal resistance scored as a "one", a rat that displays some struggle but is easy to handle would be scored as a "three" and a rat that is aggressive or otherwise difficult to remove from it's home-cage would be scored as a "six"). In general, moderate scores describe a normal rat behavior. The presence of

convulsions or tremors as a result of being handled, and the degree of palpebral closure were all scored as described above. The latency (seconds) before the animal's first step, whether or nor the animal exhibited piloerection, and general fur appearance were all recorded, as well as the total number of rears, grooming episodes, and the degree of tail elevation. The observer then began to manipulate the rat to assess: the degree of startle response elicited by a single clap (1-3, a score of two is a normal response, a score of one is no response), provoked biting elicited by placing a pencil in the rat's mouth (scored 1-5), the rat's reaction to the presence then approach of the observer's finger near the animal's head (also scored 1-5), and the rat's reaction to touch on the hindquarters (scored 1-7, with a one describing a none-responsive rat, and a seven a rat that leaps away from the observer's touch. Moderate scores from these evoked behaviors describe normal responses. Finally, the rat's gait, total gait incapacity and the degree of the animal's limb rotation (from normal stance) are recorded. Gait scores range from 1-6, with a one describing normal gait; two, a slightly ataxic gait; and six, a rat that cannot walk. Total gait incapacity describes the ability of the rat to move around despite any gait disability, gait incapacity scores range from 1-4 with a one describing normal locomotion, and a four a rat that is totally impaired and cannot walk. Limb rotation scores range from 1-5 with one describing a normal rat with no limb rotation to a five, which describes a rat with severe limb rotation.

The rats were then handled by the tail to assess their degree of positional passivity (or struggle during tail suspension), their visual placing, grip strength, and ability to grip a pencil in their forepaws. Each of these behaviors was scored by degree of severity between 1-5, (or 1-3 for the pencil-grip test). Moderate scores described normal rat behaviors. Visual placing was assessed by holding the rat suspended above the arena cage floor and then lowering the rat to the cage floor while observing the rat's forearm extension. A normal rat was scored with a value of four and displayed forearm extension well before the animal's vibrissae contacted the cage floor. Grip strength was scored as the animal's ability to grasp and hold onto the edge of the arena cage. The pencil grip tested the animal's ability to grasp a pencil placed in front of the rat as the rat was suspended by the tail above the cage floor. The presence of hypothermia or lacrimation was recorded with a value of one. Each animal was tested for extensor thrust by holding

the rat by the tail close to the arena cage bottom, and placing a hand on the pads of the animal's rear feet. Scoring ranged between 1-4. An unimpaired rat exhibits a strong push of the hind limbs against the observer's hand, and would thus be given a score of one. The non-responding, impaired rat would receive a score of four.

Pain responses were determined by quickly pinching one of the rat's hind limb toes, and their tails with blunt forceps and recording the response. The toe-pinch was scored between 1-5, and the tail-pinch between 1-7. Higher scores describe animals that are sensitive to either stimulation.

Righting reflexes were determined by flipping the animal onto it's back and recording the degree of impairment exhibited by the animal in righting itself. Similarly, the catalepsy score was determined by placing the animal's hindquarters onto a flat box 1.5 inches high in the arena cage, and recording the animal's ability to remove itself from that position. Both righting reflex and catalepsy scores range between 1-4, with a one describing the unimpaired rat, and a four a very impaired rat.

Finally, the presence of diarrhea and the degree of salivation were recorded; the animal's degree of irritability in being handled, and it's tendency to freeze were also scored. The total number of vocalizations, and the number of fecal boluses deposited in the arena cage within the three-minute test period were recorded at the conclusion of the functional observation battery.

The quantitative and descriptive data acquired during the functional observation battery were analyzed by grouping the behavioral observations into separate functional domains (Table 2).

Electrophysiology

Methods are described in our previous publication (Li et al., 2002). In addition, after baseline recording of spontaneous IPSCs from RSC pyramidal cells for 10 minutes, drugs were bath-applied in the following experiments: 1) PB (250μM) alone for 20 min., 2) memantine (300μM) alone for 20 min., or, 3) either PB (250μM) followed by memantine (300μM) co-exposure, or the reverse.

Statistical analysis

The statistics software program Origin was used to calculate each mean and standard error of the mean (SEM). Histological data is presented as mean value (+/- SEM). All raw data (the total number of FJ-B positive cells across twelve serial sections) were averaged, and the standard error of the mean determined for each treatment group. An additional one-way ANOVA with an LSD post-hoc test was done on data from the histological data using SPSS software. Behavioral data was collected onto a spreadsheet by the observer then transferred into an Excel workbook, which was constructed to re-organize the data into specific functional domains. The raw data from each functional domain at each post-treatment time-point was averaged across each treatment group and is presented as mean (+/- SEM).

Results

MK-801 + pyridostigmine bromide or (-) -physostigmine sulfate

Pyridostigmine bromide and physostigmine sulfate were tested in combination with MK-801 to determine whether these prototypical AChEIs potentiate the neurotoxicity exhibited by MK-801 alone. Reports by Olney (1991), and Corso et al., (1997) show that the neurotoxic effects of NMDA receptor antagonists are mediated by the muscarinic cholinergic system. Specifically, the muscarinic cholinergic antagonists atropine and scopolamine blocked vacuolization and HSP-70 protein induction by MK-801 in the rat cingulate cortex and retrosplenial cortex (CC/RSC) Olney (1991); Also, the addition of the non-selective muscarinic cholinergic agonist pilocarpine exacerbated the degree of cell death in the brains of PCP-exposed rats (Corso et al., (1997). The cholinesterase inhibitor, PB is not expected to cross the blood-brain barrier, although swimming stress decreased brain AChE activity in mice treated with acute doses of PB (Friedman et al., 1996), and sub-chronic PB treatment in the rat (1.85mg/kg) induced apoptosis in the cerebral cortex, striatum and hippocampus (Li et al., 2000), suggesting that under some conditions PB may access the CNS. Physostigmine has access to the

CNS and was also used in combination with MK-801 to compare results with those of PB exposed animals.

Histopathology

Animals were first injected with MK-801 (0.3mg/kg, s.c.) or 0.9% saline (s.c.) followed by a second injection of PB (0.1, 0.3 and 1.0mg/kg, i.p.) or physostigmine (0.1, 0.3 and 1.0mg/kg, i.p.). Control animals were given 0.9% saline (s.c.) followed by an i.p. injection of 0.9% saline. A total of 73 animals were used in these experiments. The two animals that received both MK-801 and the highest physostigmine dose (1.0mg/kg) both perished within the first hour after injections.

Figure 1 demonstrates the presence of FJ-B positive cells in the PC/RSC as a result of MK-801 exposure: Figure 1A shows cells from animals only exposed to MK-801 (followed by an injection of 0.9% saline), 1B and 1C show FJ-B positive cells in animals exposed to MK-801 and physostigmine (1B) or PB (1C). Figure 7 graphs the histopathological analysis of animals exposed to MK-801 and the AChEIs: PB and physostigmine sulfate. By counting FJ-B positive cells within the CC/RSC, we determined that exposure to the tested AChEI agents alone did not generate any FJ-B positive cells in the CC/RSC. Saline-injected control animals exhibited a few FJ-B positive cells: an average of 1.5 FJ-B positive cells/12 sections (+/- 0.56 SEM, n=8). As expected, exposure to 0.3mg/kg MK-801 reliably produced FJ-B positive cells in the rat CC/RSC; an average of 214.3cells/12sections (+/- 97.15 SEM, n=12). Interestingly, it appears that when combined with MK-801, the two highest PB doses may confer some protection against MK-801 induced toxicity; animals that received MK-801 and 0.3mg/kg PB had an average of 112.5 FJ-B positive cells/12 sections (+/- 18.38 SEM, n=6), and animals that received MK-801 and 1.0mg/kg PB had an average of 139 FJ-B positive cells/12 sections (+/- 59.72269 SEM, n=6).

Unlike PB, physostigmine is known to cross the blood-brain barrier, yet in our study, the presence of physostigmine in MK-801 exposed animals was not obviously neuroprotective or deleterious. Animals that received MK-801 and 0.1mg/kg physostigmine had an average of 263.7cells/12 sections (+/- 52.46 SEM n=6), and those

that received MK-801 and 0.3mg/kg physostigmine had an average of 448.3cells/12sections (+/- 257.39 SEM; n=4). A one-way ANOVA determined significant differences between groups, and a LSD post-hoc test determined that there were significant differences between the control (saline injected) animals and those injected with MK-801: and 0.1mg/kg or 0.3mg/kg physostigmine ($p>0.041$, and 0.002, respectively), or 0.1mg/kg PB ($p>0.024$).

Behavior

The behaviors that were measured by the functional observation battery were divided into several functional domains, (listed in table 2). The largest behavioral effects were exhibited by animals exposed to MK-801 with and without co-exposure to PB or physostigmine in the following functional domains: neuromuscular, pain, CNS-activity, rearing, general behavior, bizarre behavior, autonomic-GI, positional passivity, and motor affective.

Neuromuscular deficits were found in rats that were exposed to MK-801 alone and those rats that were co-exposed to MK-801, and either PB (figure 8A) or physostigmine (Figure 8B). The largest deficits were observed at the one-hour post-treatment time point, but persisted through the 24-hour post-treatment time point. Animals that were exposed only to 0.1mg/kg physostigmine also appeared to experience some neuromuscular deficit at the one-hour post-treatment time point that also persisted through the 24-hour post-treatment time point.

Animals exposed to MK-801 clearly demonstrated less pain sensitivity than either the control animals or those exposed to PB or physostigmine alone (Figures 9A and 9B). These effects persisted through the 24-hour time point.

CNS-activity increased in MK-801 exposed animals; co-exposure of MK-801 and PB or physostigmine did not ameliorate this effect, which disappeared by the 48-hour post-treatment time point (Figures 10A and 10B).

Perhaps predictably, rearing behavior in animals exposed to MK-801 was suppressed; these animals showed neuromuscular deficits that may interfere with the ability to rear. As seen in Figures 11A & 11B, in general, rearing behavior varied across

the post-treatment time period, slightly decreasing with time until the 48-hour time point when rearing behavior increased. Despite this trend, MK-801 exposed animals continued this deficit through the 48-hour time point. The addition of PB did not appear to ameliorate the rearing effect in those animals co-exposed to MK-801 (Figure 11A), however, the addition of physostigmine appeared to restore some rearing behavior in animals co-exposed to MK-801 (Figure 11B).

Neither PB nor physostigmine alone effected general behavior, which included the following parameters: palpebral closure, fur appearance, grooming, provoked biting and positional passivity. However, MK-801 suppressed general behavior during hours one and four post-treatment time, recovery began to occur by the 24-hour time point in both treatment sets (Figures 12A & 12B).

The autonomic functional domain was divided into two domains: the autonomic-general domain which included the following observations: palpebral closure, exothalmos and eye-crustiness. Animals that received 1.0mg/kg PB alone primarily demonstrated exothalmos at the four-hour post-treatment time-point, however at the 24-hour time point, animals that received MK-801 alone or in combination with PB exhibited primarily eye-crustiness (Figure 13). Animals that received MK-801 with or without co-exposure to physostigmine only exhibited eye-crustiness at the 24-hour time point (data not shown). The autonomic-GI functional domain only records the total number of fecal boluses produced by the animal during the three-minute observation period in the arena setting. Exposure to MK-801 alone reduced the number of fecal boluses at the one and four-hour post-treatment time-points; animals co-exposed to MK-801 and PB or physostigmine also had reduced bolus production at the four-hour post-treatment time-points with recovery by 24-hours post-treatment (data not shown).

In order to unmask specific behaviors that were primarily affected by drug exposure, both bizarre behavior and positional passivity were each measured separately from the general behavior domain. Exposure to MK-801 with or without co-exposure to the AChEIs, PB or physostigmine had a variable but large effect on bizarre behavior, which was primarily exhibited by head-weaving and aimless wandering behaviors (figures 14A & 14B). Any exhibition of bizarre behavior disappeared by the 24-hour post-treatment time-point. Animals exposed to MK-801 demonstrated a profound deficit

in the positional passivity domain through the 48-hour time-point; neither the presence of PB or physostigmine in these animals ameliorated this deficit (Figures 15A & 15B). Exposure to the AChEIs alone had no effect on positional passivity.

Motor-affective behavior was assessed by measuring the time to first movement, the animal's grasp irritability, provoked freezing behavior and the number of vocalizations produced by the animal during the three-minute examination period. The time to first movement by the animal after it was moved from its home cage to the arena was the primary factor in the total motor-affective score. Exposure to the AChEIs PB or physostigmine alone did not have an effect on motor-affective behavior (Figures 16A & 16B). Exposure to MK-801 with or without co-exposures to PB or physostigmine increased the motor-affective deficit through the one and four-hour post-treatment time-points. Animals appeared to recover this deficit by the 24-hour time-point.

The behavioral results from the remaining functional domains did not illuminate any specific drug-induced deficits. Surprisingly, the sensorimotor functional domain which records the rats startle response and righting reflex as well as a variety of escape responses did not show any consistent drug effects (data not shown). There were also no strong drug-induced effects by animals in the CNS-excitability functional domain, which measures convulsions and tremors exhibited by animals in the arena after they were moved from the home-cage environment (data not shown).

NMDA receptor antagonists: dextromethorphan, felbamate and memantine with and without co-exposure to the AChEI, pyridostimine bromide (PB)

Based on the histological results from the experiments described above, it appeared that there was some interaction between the NMDA receptor antagonist, MK-801 and the AChEI, PB, and a variable interaction between MK-801 and physostigmine. Specifically, at higher doses (0.3 and 1.0mg/kg), PB appeared to be neuroprotective when animals were co-exposed to 0.3mg/kg MK-801 (Figure 7). Also, when given alone, PB did not induce any FJ-B positive cells in the CC/RSC. Thus, we elected to choose the highest tolerable PB dose that we tested (described in above experiments) in combination with a series of other NMDA receptor antagonists; we chose antagonists that may be

clinically encountered to determine whether the combinations of these drugs with PB may be neuroprotective, or may instead induce neurotoxicity in the rat CC/RSC. We began these experiments by sequentially testing each proposed NMDA receptor antagonist at the highest proposed dose with and without co-exposure to 1.0mg/kg PB.

Histopathology

Animals were first injected with the NMDA receptor antagonist (i.p.) or 0.9% saline (i.p.) followed by an injection of PB (1.0mg/kg, i.p.). Control animals were given 0.9% saline (i.p.) followed by a second i.p. 0.9% saline injection. A total of 65 animals were used in these experiments. There were several deaths, of the three animals that received both PB and 50mg/kg dextromethorphan, two died within an hour after their injections. Also, co-exposure of memantine and PB (1.0mg/kg) proved lethal for many animals within an hour after injection: at 75mg/kg memantine, 3/6 animals died, at 50mg/kg memantine, 1/3 animals died, at 37.5mg/kg, 4/5 animals died, and at 25mg/kg memantine, 2/7 animals died. The rats that perished expressed facial tremors and seizures within ten minutes after injections. In an effort to elucidate whether activation of the muscarinic cholinergic receptors by PB may have contributed to the death of memantine/PB co-exposed animals, a few animals were pre-injected with atropine sulfate (s.c.) at 25.0mg/kg (Li et al., 2000). The animals that received atropine sulfate in combination with 37.5mg/kg or 25mg/kg memantine and 1.0mg/kg PB, or 25mg/kg memantine and saline survived. None of the animals that received memantine alone at any dose died (n=1 each treatment).

The antitussive NMDA receptor antagonist dextromethorphan did not produce any FJ-B positive cells in the CC/RSC when tested at the highest proposed dose (50mg/kg), when administered alone or in combination with PB. However, the highest dextromethorphan dose in combination with PB was lethal for 2/3 rats that received both drugs.

We tested the anti-convulsant felbamate at the highest proposed doses (400mg/kg and 300mg/kg) alone and in combination with PB. No neurodegeneration was detected, nor was this a lethal combination.

We tested the anti-parkinson, non-competitive NMDA receptor antagonist, memantine at several doses, those initially proposed: 25, 50 and 75mg/kg, as well as at 0.36mg/kg (to represent a typical therapeutic dose- 10mg twice/day), and finally at 37.5mg/kg (approximately 100X therapeutic dose). We determined that at all doses except the lowest (0.36mg/kg), memantine alone causes a variable degree of neurodegeneration in the CC/RSC. Figure 2A shows 10X photographs of FJ-B positive cells in animals exposed to MK-801 alone (0.3mg/kg) or to memantine alone (75mg/kg) in the PC/RSC region. Figure 3 shows 25X photographs of FJ-B positive cells in animals co-exposed to 1.0mg/kg PB and 75mg/kg memantine 3A or given 75mg/kg memantine alone (Figure 3B). Figure 4 25X shows photographs of FJ-B positive cells in animals co-exposed to 1.0mg/kg PB and 50mg/kg memantine (Figure-4A) or exposed to 50mg/kg memantine alone (Figure 4B). Figure 5 shows 25X photographs of FJ-B positive cells in animals co-exposed to 1.0mg/kg PB and 36.5mg/kg memantine (5A) or given 36.5mg/kg memantine alone (figure 5B). Figure 6 shows 25X photographs of FJ-B positive cells in animals co-exposed to 1.0mg/kg PB and 25mg/kg memantine (6A) or exposed to 25mg/kg memantine alone (Figure 6B).

As shown in (Figure 17), the mean number of FJ-B positive cells increased as the memantine dose increased. Perhaps the most striking result was that the co-administration of 1.0mg/kg PB to memantine increased the number of FJ-B positive cells. Figures 18A and 18B further illustrate the degree to which PB adds to the neurotoxic effect of memantine; these figures include the data from treatment groups with multiple animals (treated or surviving treatment) at either 25mg/kg or 75mg/kg. Animals exposed to 25mg/kg memantine alone had an average of 24.75 FJ-B positive cells/12 sections (+/- 7.65 SEM, n=5), animals co-exposed to 25mg/kg memantine and PB had an average of 42 FJ-B positive cells/12 sections (+/- 13.49 SEM, n=5), (Figure 18A). Animals exposed to 75mg/kg memantine alone had an average of 218.67 FJ-B positive cells/12 sections (+/- 112.14 SEM, n=3), animals co-exposed to 75mg/kg memantine and PB had an average of 1171.33 FJ-B positive cells/12 sections (+/- 654.74 SEM, n=3), (Figure 18B). One-way ANOVA detected significant differences between groups. An LSD post-hoc analysis determined significant differences in the number of positive FJB cells between animals co-exposed to PB and 75mg/kg memantine and: saline control animals, animals

exposed to 25 and 75mg/kg memantine alone, and animals co-exposed to PB and 25mg/kg memantine ($p > 0.001$, $p > 0.002$, $p > 0.002$ and $p > 0.011$, respectively).

Animals that received an atropine pre-injection before injection with 25mg/kg memantine and either saline or PB had no positive FJ-B cells (n=1, each treatment). Also, the single animal that received atropine prior to injection with 37.5mg/kg memantine and PB had no positive FJ-B cells.

Behavior

The functional domains that were affected by felbamate exposure include: CNS-activity, autonomic-general and GI, bizarre behavior and motor-affective behaviors Exposure to dextromethorphan did not reveal many changes in behavior, with the exception that rearing behavior was suppressed at the one-hour time point (figures not shown). However, co-exposure to PB and felbamate (400mg/kg) affected the animals rearing, autonomic-general and GI behaviors. Co-exposure of PB to dextromethorphan did not have an additive behavioral effect to that by dextromethorphan alone (figure not shown).

Animals exposed to memantine demonstrated changes in the following functional domains: neuromuscular, sensorimotor, pain, CNS-excitability, CNS-activity, rearing, autonomic-GI, bizarre behavior, and positional passivity. Animals exposed to doses 25mg/kg and greater experienced increased neuromuscular deficits through the four-hour post-treatment time-point, and recovered by 24-hours; animals co-exposed to PB did not exhibit increased neuromuscular deficits over those exposed to memantine alone, nor was a dose-response effect evident (Figure 19).

The sensorimotor behaviors are affected by high dose (75mg/kg) memantine (Figure 20). A deficit in sensorimotor behaviors occurred in animals exposed to 75mg/kg memantine that persisted through the four-hour time-point; PB co-exposure does not exacerbate this effect.

Not surprisingly, given the therapeutic nature of memantine, the highest two doses (50 and 75mg/kg) produced reduced pain response in the animals that persisted through the four-hour post-treatment time-point (figure not shown).

Although there was a great degree of variability, CNS-excitability was increased in animals exposed to 75mg/kg memantine; this effect persisted through the four-hour time-point and was not further affected by co-exposure to PB (Figure 21). CNS-activity behaviors were suppressed by exposure to 25mg/kg memantine with and without co-exposure to PB (Figure 22).

Rearing behavior was also suppressed by memantine exposure, particularly at the one-hour post-treatment time-point; this effect was variably ameliorated by PB co-exposure, and persisted through the 24-hour time-point (Figure 23).

Animals exposed to memantine alone produced fewer fecal boluses; this effect was ameliorated by co-exposure to PB, and only persisted through the four-hour time-point (figure not shown).

Bizarre behavior was variably expressed by animals exposed to memantine doses 25mg/kg and greater. Most of the observed bizarre behaviors were described as head shaking, crawling, aimless wandering and rearing (figure not shown).

Finally, the animals' positional passivity was also affected by exposure to memantine; co-exposure to PB slightly exacerbated this effect. Most of the animals recovered normal struggle behavior during the passivity test by the 48-hour post-treatment time-point, however, animals co-exposed to high-dose (75mg/kg) memantine and PB experienced a deficit through 48-hours (Figure 24).

The presence of bizarre behavior in the animals injected with 25-75mg/kg memantine was predictive of positive FJ-B cells in the rat CC/RSC; for example, of the 26 surviving animals, 18 expressed both bizarre behavior and positive cells (69%). 19% of the surviving animals expressed no bizarre behavior or exhibited any detectable neurodegeneration; however, all survivors that received 75mg/kg memantine expressed FJ-B positive cells in the CC/RSC.

Electrophysiology: Co-exposure of Pyridostigmine bromide and memantine in cortical pyramidal cells.

We examined the effect of PB alone on sIPSCs from six RSC pyramidal cells. Overall, PB alone has no effect (n=3) or only produces slight reduction of sIPSCs (n=3,

$p>0.05$, K-S test. Memantine significantly reduced sIPSCs in only two cells ($p<0.05$, K-S test). There was no effect of memantine on the remaining cells tested (n=4). However, when co-applied, 8/12 pyramidal cells showed a profound reduction of sIPSPs, while the remaining cells did not respond to the drugs. Figure 25 shows a cumulative response of sIPSCs of a pyramidal cell after sequential administration of memantine and PB.

Discussion

The present study was designed to investigate the neurotoxic risks that are associated with the combined use of acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor antagonists. This is a particularly relevant topic-in that many people encounter AChEIs and NMDA antagonists in a wide range of settings. AChEI's exist as therapeutic drugs, insecticides, and as prophylactic agents against nerve gas poisoning. Drugs that posses NMDA antagonist activity include currently available therapeutic drugs (e.g. certain anticonvulsants and antitussives) and also as investigational drugs (e.g., drugs for Parkinson's disease, spasticity, chronic pain, dementias, and anticonvulsants effective against severe nerve gas-induced seizures). NMDA antagonist activity is also present in several drugs of abuse, including ethanol or phencyclidine (PCP, or angel dust). It is increasingly likely that agents from both of these groups will be co-administered, with potentially dangerous consequences, especially in life-threatening situations such as exposure to - or treatment for - nerve gas poisoning.

The most important results of this study demonstrate that in mature (retired breeder) female rats, the NMDA receptor antagonist memantine caused neurodegeneration in the PC/RSC. This effect was further exacerbated by co-exposure to the AChEI, PB. Thus, as hypothesized in our original proposal, there is a synergistic neurotoxic effect due to co-exposure to an AChEI and an NMDA antagonist. Furthermore, this interaction could have wide-ranging implications, for PB is important for nerve gas prophylaxis, and memantine for Parkinson's and chronic pain.

Proposed Mechanisms of Neurotoxicity

Neurons in the PC/RSC are especially at risk to increased extracellular concentrations of glutamate, the brain's principle excitatory neurotransmitter (Olney 1989). In normal, non-pathogenic states, extracellular glutamate levels are modulated by reuptake mechanisms- even so, extracellular concentrations of glutamate are close to concentrations that also promote an excitotoxic response in susceptible neurons (reviewed by Lipton and Rosenburg, (1994)). Thus, any perturbations that increase glutamate concentration within a synapse may also incite an excitotoxic response in surrounding neurons. For example, increased concentrations of glutamate (an NMDA receptor agonist) can activate surrounding NMDA receptors to such a degree that intracellular excitotoxic mechanisms may be initiated. This process has been implicated in many neurological conditions. Stroke is an example of a pathological event that leads to persistent, excess glutamate in the synapse, and may lead to subsequent cell death. Military neurotoxins that cause prolonged seizures present another situation in which endogenous glutamate can result in extensive neuronal damage and death.

When we first proposed that this neurotoxic interaction was possible, the mechanisms underlying the interaction were still speculative. However, one possible mechanism involved disinhibition. Specifically, it was possible that the two classes of drugs produced an imbalance between neuronal excitation and inhibition such that inhibition was reduced, and this resulted in excessive hyperexcitability sufficient to cause excitotoxicity. For example, based on the fact that NMDA receptors can mediate tonic inhibition in cortical neurons, John Olney and colleagues proposed the following mechanism: glutamate released from PC/RSC neurons binds to NMDA receptors expressed on nearby GABAergic neurons, the GABAergic neurons in turn act to inhibit ACh release onto M1 muscarinic receptors in PC/RSC neurons, the net effect of which is to suppress cholinergic stimulation (Olney et al., 1991). Blockade of NMDA receptors (by both competitive and non-competitive antagonists) on GABAergic neurons abolishes the inhibition of ACh at PC/RSC neurons, and thus exposes these cells to a state of cholinergic hyperstimulation. Olney (1991) confirmed the role the cholinergic system has in mediating glutamate release by cortical neurons, by demonstrating that the

excitotoxic effect of NMDA receptor blockade can be ameliorated by the muscarinic cholinergic antagonist, atropine. Olney also determined that the muscarinic agonist pilocarpine exacerbates excitotoxicity in susceptible neurons (Corso et al., 1997).

There are several examples of normal cortical physiological functions that are altered by NMDA receptor antagonists, such as: cortical glucose metabolism (Kurumaji et al., 1989; McCulloch and Iverson 1991), heat-shock protein induction (Sharp et al., 1991; Berger et al., 1994; Corso et al., 1997; Lan et al, 1997), and cortical neuron vacuolization and necrosis (Olney 1989; Ellison and Switzer 1993; Hargreaves et al., 1993; Fix 1994; Fix et al., 1993).

AchIEs

We chose to use physostigmine and PB in our preliminary experiments to test whether co-exposure of these AChEIs with the NMDA receptor antagonist MK-801 resulted in increased excitotoxicity. Our later studies specifically investigated the effect that PB may have in inducing neurotoxicity in PC/RSC neurons after they are co-exposed to various NMDA receptor antagonists that may be clinically or militarily encountered. Physostigmine was used as a representative AChEI that has activity in the central nervous system (CNS), as opposed to PB, which is primarily active in the peripheral nervous system (PNS). PB is a topically relevant AChEI, as it may be used as a prophylactic agent against nerve agents in future wars. PB is a quaternary dimethyl carbamate that is used therapeutically to treat myasthenia gravis, and was also given to military personnel during the Gulf War (Persian Gulf Veterans Coordinating Board, 1995). Unlike physostigmine, PB is not expected to cross the blood-brain barrier- due to the positive charge on the quaternary nitrogen atom (Birtley et al., 1966). However, a study by Friedman et al., (1996) suggests that under conditions of stress (forced swimming stress in mice) PB may cross the blood-brain barrier to inhibit AChE in the brain. A 90mg/day dose (30mg dose, taken three times/day) of PB temporarily inhibits 30-40% of the AChE in the peripheral nervous system (Blick et al., 1991), toxic exposure to PB results from overstimulation of the muscarinic and nicotinic cholinergic systems in the periphery.

We chose to use older female rats for our studies, to enhance an excitotoxic response- as both the sex and age of the animal mediates the excitotoxic effect of NMDA receptor antagonists (Fix 1994; Auer 1996; Farber et al., 1995; Guo-Ross et al., 2002).

MK-801 +/- AChEIs (physostigmine or PB)

We found that low dose MK-801 (0.3mg/kg) consistently produced FJ-B positive cells in the PC/RSC, although there was quite a bit of variability between animals in the numbers of positive cells. We also found that (0.3mg/kg) physostigmine increased the number of positive FJ-B cells in animals co-exposed to MK-801. Thus, as expected, NMDA receptor-blockade by MK-801, combined with increased ACh concentrations (by physostigmine co-exposure) produced on average, more FJ-B positive cells than did MK-801 exposure alone. We then tested whether PB produced a similar degree of neurotoxicity in animals co-exposed to MK-801. We found that when combined with a low dose of MK-801, PB may be somewhat neuroprotective, as fewer FJ-B cells were evident on average in the co-exposed animals. This finding was consistent with our initial preliminary results using FJ, in which modest doses of PB may actually be neuroprotective against MK-801 neurotoxicity. Thus, it is clear that blanket statements cannot be made about all drugs in a particular group, and only with careful and detailed studies are the actual interactions revealed.

Thus, PB appears to ameliorate the excitotoxic effect of MK-801 in mature female rats. We then tested a series of additional NMDA receptor antagonists to establish whether they may induce excitotoxicity in the PC/RSC, and additionally, whether co-exposure to PB may ameliorate or exacerbate a toxic response. We chose to investigate the NMDA receptor antagonists: felbamate, dextromethorphan and memantine. All of these therapeutic drugs have been described as neuroprotective compounds in either rat focal ischemia models (Wasterlain et al., 1992; Britton et al., 1997), or induced traumatic brain injury in the rat (Rao 2001); both are conditions that induce glutamate-mediated injury to cortical cells surrounding the experimental insult.

Felbamate +/- AChEIs

Felbamate is used as an anticonvulsant agent for epilepsy. Felbamate has the properties of a non-competitive NMDA receptor antagonist, but its exact site of action on the NMDA receptor is a matter of dispute. It has been proposed to bind within the ion channel (Rho et al., 1994), at the glycine site (Koek & Colpaert 1990), or none of the above (White et al., 1992). Our histological results did not indicate that felbamate had any excitotoxic effects on cells in the PC/RSC; nor did we detect any interaction between felbamate and PB.

Dextromethorphan +/- AChEIs

Dextromethorphan is an antitussive non-competitive NMDA receptor antagonist. However, it is not currently known whether neuroprotection by dextromethorphan against glutamate toxicity is primarily due to a direct interaction with the NMDA receptor (Britton et al., 1997). Our results did not suggest that dextromethorphan initiated an excitotoxic response by PC/RSC cells. We also did not detect any interaction between dextromethorphan and PB in co-exposed animals.

Memantine +/- AChEIs

Memantine is used clinically in United States for Parkinson's disease (reviewed by Kornhuber and Weller 1996), and is in phase three trials for clinical use against vascular dementias (Orgogozo 2002). Memantine is a non-competitive antagonist that binds within the NMDA receptor ion channel (Kornhuber et al., 1989; Bormann 1989; Parsons et al., 1993). Memantine induces heat-shock protein in the PC/RSC and also the dentate gyrus (Tomitaka et al., 1996); this result is similar to others in which non-competitive NMDA receptor antagonists induced heat shock proteins in the PC/RSC, for example phencyclidine (PCP) (Corso et al., 1997), MK-801 (Berger et al., 1994), dextrorphan, (Lan et al., 1997), and ketamine (Sharp et al., 1991). Thus, we were not surprised to find that memantine exposure produced an excitotoxic response, as demonstrated by the presence of FJ-B positive cells in the PC/RSC.

We were surprised that co-exposure to PB exacerbated the neurotoxicity exhibited by memantine, particularly since this effect opposes that which we saw previously, in which PB appeared to ameliorate MK-801 toxicity in the PC/RSC. Given that both memantine and PB are therapeutically used drugs, this is a particularly troubling effect. It is important to note that although most of the memantine doses used in our experiments were atypically high, due to differences in metabolism between rats and humans, the acute moderate dose we used (25mg/kg) is only modestly higher than the equivalent dose in the human (reviewed by Parsons, et al, 1999). At 25mg/kg memantine, we noted striking variabilities between animals in the degree of expressed neurotoxicity- this variability ranged from very few positive FJ-B cells in animals exposed to memantine alone, to death in some animals co-exposed to memantine and PB (2/7 co-injected animals died); the surviving co-injected animals all expressed FJ-B positive cells in the PC/RSC, suggesting that PB does interact with memantine to induce a neurotoxic response in these cells. Our results also indicate that the 25mg/kg dose in the older female rat is near a threshold of excitotoxicity. Perhaps due to natural variations between animals, some animals appeared to be more sensitive than others to memantine. However, the addition of PB appeared to prime the level of sensitivity in many animals, creating a neurotoxic reaction in co-exposed animals. All higher doses (37.5-75mg/kg) of memantine we tested consistently produced FJ-B positive cells in the PC/RSC, and this effect was exacerbated by PB co-exposure.

Based on results by Tomitaka et al., (1996), who showed that memantine induced heat-shock protein both in the PC/RSC and the dentate gyrus, we also examined the dentate gyrus in our surviving high dose animals (75mg/kg) that were co-exposed to PB, and did not note the presence of FJ-B positive cells. This result indicates that specific neurons within PC/RSC are uniquely sensitive to non-competitive NMDA receptor antagonists, thus supporting Olney's hypothesis regarding this vulnerable neural population (Olney et al., 1991).

Behavioral Data from the FOB Tests

Our behavioral data support our histological data with respect to the neurotoxic effects of NMDA receptor antagonists on PC/RSC neurons. Specifically, both of the non-competitive, voltage-sensitive NMDA receptor antagonists that we investigated (MK-801 and memantine) caused profound stereotypical neurobehavioral effects that usually corresponded to neurotoxic damage in the PC/RSC. In addition to inducing head-weaving behavior, we found that both MK-801 and memantine increased locomotor stimulation in exposed rats; we scored this behavior as "aimless wandering". This is similar to results by Bubser et al., (1992), who suggested that the stimulatory behavior of MK-801 and memantine is a general result of increased dopamine levels in the nucleus accumbens by NMDA receptor non-competitive antagonists. Both MK-801 and memantine induced comparable neuromuscular deficits and similarly: they suppressed rearing behavior, positional passivity and CNS activity scores. MK-801 had more profound effects on autonomic, motor-affective and general behaviors than did memantine, however memantine had a greater effect on suppressing sensorimotor and CNS excitability scores. Although MK-801 and memantine are both voltage sensitive non-competitive NMDA receptor antagonists, it is not surprising that there are some differences in behavior- as suggested by Danysz et al., (1994), the observed differences in behavior in animals exposed to various NMDA receptor antagonists suggest that these drugs may act at sites additional to those on the NMDA receptor.

Of the three non-competitive NMDA receptor antagonists we tested with co-exposure to PB, only memantine produced an excitotoxic response in the PC/RSC. These results suggest that in general, non-competitive antagonists acting within the ion channel may be more likely to induce an excitotoxic response leading to cell death in the PC/RSC, and that non-competitive NMDA receptor antagonists that are not known to block the ion channel are not as toxic. However, Hargreaves et al., (1993) examined changes in rat neuronal morphology produced by exposing the animals to either competitive or non-competitive NMDA receptor antagonists. Based on the presence of vacuolized cells and altered glucose metabolism, Hargreaves concluded that the functional consequences of NMDA receptor blockade are the same regardless of where on the receptor the antagonist binds. Despite this conclusion, it must be noted that the

animals in the Hargreaves study were only allowed to survive four hours after they were dosed; thus, any progression of excitotoxic mechanisms may not have been appreciated.

Our results regarding the excitotoxic nature memantine contradict the apparent safety and efficacy of this drug. As reviewed by Parsons et al., (1999), memantine has been well tolerated and in use clinically for over 15 years. Memantine is an open channel blocker (Chen, et al, 1992), therefore it only blocks the NMDA receptor after the cell depolarizes; and, due to its low affinity, the next depolarization allows memantine to leave the ion channel. It is thought that the rapid blocking and unblocking action of memantine inhibits the overall activity of NMDA receptors, yet leaves enough receptors unblocked at any one time to facilitate cognitive function, and to also suppress cholinergic function enough to avoid a neuronal excitotoxic response. Chen et al., (1998) hypothesized that memantine is most effective during pathological conditions that result in increased extracellular glutamate concentrations- to suppress the resulting cholinergic hyperactivation of neurons (Chen et al., 1998).

Our results demonstrate that PB appears to differentially ameliorate (in the case of MK-801 co-exposure) or exacerbate (in the case of memantine co-exposure) NMDA receptor non-competitive antagonists that bind within the receptor's ion channel. This effect may help elucidate a possible cholinergic mediated model for memantine-induced toxicity in the PC/RSC. This differential effect also suggests that PB and memantine may interact to increase the potential for a neurotoxic response. If one supposes that PB remains in the PNS, then cholinergic hyperactivity in the periphery may be detected by CNS mechanisms, leading to cholinergic hyperactivity in the brain.

Chaney et al., (1999) described experiments in which PB induced seizures in mice that could not be suppressed by centrally acting anticonvulsants, yet the seizures were inhibited by the muscarinic antagonist, atropine. Li et al., (2000) determined that PB-mediated apoptosis in the rat could be inhibited by pre-exposure of the animals to atropine, again suggesting that PB has CNS effects. Given that therapeutic use of memantine increases ACh in the CNS (mediated by NMDA receptor blockade), the addition of an AChEI agent (PB) may further increase ACh concentrations to induce hyperactivity in cholinergic neurons. Our results support the current hypotheses that there is a strong cholinergic component to the initiation of excitotoxicity in PC/RSC

neurons: Co-administration of MK-801 and physostigmine increased the average number of FJ-B cells in this region over that of MK-801 exposure alone. However, PB appeared to be neuroprotective against MK-801 toxicity. Perhaps some concentration of PB crosses the blood-brain barrier to inhibit the activity of MK-801 directly; PB may also interact directly with memantine- however, this interaction appears to be excitotoxic.

Electrophysiological Pilot Study: Memantine + PB

Our electrophysiological studies suggest that indeed, a potential interaction may occur between memantine and PB; when the two drugs were bath co-applied to brain slices, 66% (8/12) of the pyramidal cells tested showed more profound reduction of sIPSCs, while the remaining pyramidal cells did not respond to the drugs.

Therapeutic Implications

There is current interest in combining memantine and AChEIs therapeutically to treat Alzheimer's disease (AD) (Wenk et al., 2000). AD is associated with the over-activation of NMDA receptors and the subsequent loss of cholinergic neurons. Thus, treatment includes increasing concentrations of ACh by inhibiting AChEs, as well as by decreasing the hyperactivation of NMDA receptors. By combining these compounds, the hope is to ameliorate the cognitive deficits of AD and slow disease progression by sparing cholinergic neurons from glutamate insult. However, in a vulnerable population, one might expect some neurotoxic response to memantine. Indeed, cases of pharmacotoxic psychosis in Parkinson's disease patients induced by memantine have been reported (Riederer et al., 1991). Futhermore, since the brain areas most affected are involved in subtle behaviors and integrative processes - damage to these areas may not be as obvious as, for example, damage in the motor cortex where a limb could become non-function, as occurs after a stroke. Thus, an individual with advanced Parkinson's or Alzheimers to whom such drugs may be given could have difficulty sensing the outcome of neurological damage. Similarly, their physicians and care-givers might simply assume

that drug-induced changes in neurological status are merely a progression of the disease, and may not suspect that a new decline is from a neurotoxic drug interaction.

Factors Influencing Memantine Neurotoxicity: Age, Gender, and Genes

Vulnerability to memantine may be a function of age, sex or genetic variability. For example, Farber et al., (1995) suggested that increased NMDA receptor hypofunction in the rat is an age-related result, therefore, it is possible that too many of the remaining functional NMDA receptors are inhibited by a standard dose of memantine. Serra, et al, (1994) also determined that although there are fewer NMDA receptors in the aged rat, both glutamate and glycine increased the ability of the NMDA receptor to bind MK-801. This suggests that pathological conditions that increase extracellular glutamate levels may also increase memantine's affinity to bind the NMDA receptor, leaving too few NMDA receptors unblocked and thus lead to an excitotoxic reaction.

Sex probably also plays a role in increased vulnerability to NMDA receptor antagonists (Fix 1994; Auer 1996; Farber et al., 1995; Guo-Ross et al., 2002). For example, results by Smith, (1989) determined that estrodiol modulates NMDA receptor binding by potentiating binding of the receptor to the agonist, NMDA. This also suggests a possible mechanism for neuronal overexcitation- if the receptors are already slightly hyperactive (by estrogenic activity) and then are confronted with memantine, too many receptors may be blocked to facilitate cholinergic inhibition to cortical neurons.

Finally, genetic variability may also determine who may be more at risk for NMDA receptor mediated neurotoxicity. Therefore, people who are atypical butyrylcholinesterase carriers and exhibit a strong cholinergic response to AChEIs, may also react negatively when co-exposed to non-competitive NMDA receptor antagonists (Loewenstein-Lichenstein et al., 1995).

Potential Future Studies

Future studies should take several approaches to elucidate a mechanism that describes how memantine exposure induces an excitotoxic reaction in rats. First, it

would be instructive to determine whether the CNS-acting AChEI physostigmine exacerbates memantine cytotoxicity; we predict that it will. Additional investigations of other commonly encountered carbamate AChEIs in animals co-exposed to memantine should be completed to determine whether indeed, memantine attenuates their activity in the CNS as we propose may occur in an interaction between PB and memantine. Both the age and the sex of the animal should be investigated with respect to memantine toxicity, adolescent male and female animals should be investigated, as well as should older males. These groups all have relevance to military populations, for young military personnel may be more likely to be in combat situations and at risk of exposure to nerve gas agents, whereas higher officers, and retired veterans are among those most likely to be candidates AChEI-type anti-Parkinson's and AD therapies.

Although our initial pilot electrophysiological findings also suggest that there is a tendency for the combination of memantine and PB to result in additive depressive action on iSPSCs in the cortex, further experiments are required to explore the possible correlation of combined exposure of these compounds to neuronal death.

Key Research Accomplishments

1. The histopathological effect of the non-competitive NMDA antagonist MK-801 is ameliorated by co-exposure to the acetylcholinesterase inhibitor, pyridostigmine bromide in retired breeder rats; however neurotoxicity is exacerbated by co exposure to the AChEI, physostigmine.

2. The NMDA receptor antagonists: dextromethorphan and felbamate do not induce neurotoxicity in exposed animals, nor does co-exposure of these compounds to pyridostigmine bromide induce detectable neurotoxicity.

3. The NMDA receptor antagonist, memantine induces a neurotoxic response visualized by positive Fluoro-Jade-B stain in mature female rats; this effect is exacerbated by pyridostigmine bromide.

4. The resultant neuropathology in MK-801 and memantine exposed animals is in good agreement with the behavioral deficits exhibited by animals exposed to these compounds.

5. Combined exposure of memantine and PB had a greater effect on recorded sIPSPs than did memantine or PB alone.

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Appendix

Figure legends

Table 1: Functional observation battery test panel. The observer used a test panel to sequentially test and score behaviors of interest at each time-point pre and post-drug treatment. The observer first scored home cage observations (behaviors 1-8), followed by non-interactive, arena observations (behaviors 9-18). The scoring of the remaining behaviors relied increasingly on interactions with the observer, for example, positional passivity and extensor thrust observations.

Table 2: Functional behavioral domains. The results scored on the test panel were entered into a computer spreadsheet designed to re-organize the data into functional domains. This enabled us to score multiple behaviors for each domain in the least obtrusive manner possible. In this manner, 45 observed behaviors at each time-point were re-oganized into 11 functional domains at each time-point.

Figure 1: Fluoro-Jade-B positive cells in the cingulate and retrosplenial cortecies of adult female rats: FJ-B staining of coronal brain sections of PC/RSC region from an adult female rat that was exposed to: A) MK-801 (0.3mg/kg) followed by 0.9% saline, B) MK-801 (0.3mg/kg) followed by physostigmine (0.3mg/kg), or C) MK-801 (0.3mg/kg) followed by PB (1.0mg/kg). The photographs show FJ-B positive cells (bright green) in layer III of the RSC. Photographs were taken at 25X magnification using a water immersion lens.

Figure 2: Comparison of FJ-B positive cells in the cingulate and retrosplenial cortecies of adult female rats induced by the non-competitive NMDA receptor antagonists, MK-801 and memantine. FJ-B staining of coronal brain sections of PC/RSC region from an adult female rat three days after exposure to: A) MK-801 (0.3mg/kg) followed by 0.9% saline, B) memantine (75mg/kg) followed by 0.9% saline. These 10X photographs show FJ-B positive cells (bright green) in layer III of the RSC.

Figure 3: Comparison of FJ-B positive cells in the cingulate and retrosplenial cortecies of adult female rats induced by: A) 75mg/kg memantine and saline or, B) co-exposure to 75mg/kg memantine and PB. Photographs were taken at 25X magnification using a water immersion lens.

Figure 4: Comparison of FJ-B positive cells in the cingulate and retrosplenial cortecies of adult female rats induced by: A) 50mg/kg memantine and saline or, B) co-exposure to 50mg/kg memantine and PB. Photographs were taken at 25X magnification using a water immersion lens.

Figure 5: Comparison of FJ-B positive cells in the cingulate and retrosplenial cortecies of adult female rats induced by: A) 37.5mg/kg memantine and saline or, B) co-exposure to 37.5mg/kg memantine and PB. Photographs were taken at 25X magnification using a water immersion lens.

Figure 6: Comparison of FJ-B positive cells in the cingulate and retrosplenial cortecies of adult female rats induced by: A) 25mg/kg memantine and saline or, B) co-exposure to 25mg/kg memantine and PB. Photographs were taken at 25X magnification using a water immersion lens.

Figure 7: Graph of histological results. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average number of FJ-B positive cells across 12 serial sections (approximately every 6^{th} section) +/- SEM.

Figure 8: Effect of AChEIs and MK-801 on neuromuscular behaviors. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-

physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the following behaviors: tail elevation, tail elevation, gait, limb rotation, grip and extensor thrust. **A**. Neuromuscular behavioral data from adult female rats exposed to MK-801 and/or PB. **B**. Neuromuscular behavioral data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 9: Effect of AChEIs and MK-801 on pain sensation. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the degree of response to toe and tail pinches with blunt forceps. **A**. Pain sensory data from adult female rats exposed to MK-801 and/or PB. **B**. Pain sensory data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 10: Effect of AChEIs and MK-801 on CNS-activity. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the following home-cage observations: body posture, tremors, twitches and the presence of convulsions. **A**. CNS-activity data from adult female rats exposed to MK-801 and/or PB. **B**. CNS-activity data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 11: Effect of AChEIs and MK-801 on rearing behavior. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by keeping a tally of the total number of rears made in the first three minutes in the arena.

A. Rearing data from adult female rats exposed to MK-801 and/or PB. **B**. Rearing data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 12: Effect of AChEIs and MK-801 on general behavior. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the following behaviors: palpebral closure, fur appearance, total number of grooming episodes (within first three minutes in arena) and provoked biting. **A**. General behavior in adult female rats exposed to MK-801 and/or PB. **B**. General behavior in adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 13: Effect of pyridostigmine bromide and MK-801 on autonomic observations. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or were co-injected with both MK-801 and PB. Bars plot the average scores (+/- SEM) from raw data obtained by observation of palpebral closure (in home cage), exothalmus, eye-crustiness, piloerection, hypothermia and lacrimation.

Figure 14: Effect of AChEIs and MK-801 on bizarre behavior. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the presence of odd behaviors, for example aimless wandering, or head-weaving behaviors. **A**. Bizarre behavior data from adult female rats exposed to MK-801 and/or PB. **B**. Bizarre behavior data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 15: Effect of AChEIs and MK-801 on positional passivity. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by scoring the degree of the rats struggle when briefly suspended by the tail close to the arena cage floor. **A**. Positional passivity data from adult female rats exposed to MK-801 and/or PB. **B**. Positional passivity data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 16: Effect of AChEIs and MK-801 on motor-affective behavior. Rats were treated with injections of saline (control), saline co-injected with either MK-801 (0.3mg/kg), pyridostigmine bromide (0.1, 0.3 and 1.0 mg/kg), or (-)-physostigmine (0.1, 0.3 mg/kg); other rats were co-injected with both MK-801 and PB or with MK-801 and (-)-physostigmine. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the rats arousal and general irritability when handled, as well as the time (in seconds, timed up to 180 seconds) before the rat voluntarily moved after being placed in the arena cage from the home cage. **A**. Motor-affective data from adult female rats exposed to MK-801 and/or PB. **B**. Motor-affective data from adult female rats exposed to MK-801 and/or (-)-physostigmine.

Figure 17: Fluoro-Jade-B positive cells in the cingulate and retrosplenial cortecies of adult female rats treated with the non-competitive NMDA receptor antagonist, memantine and co-injected with either saline or PB (1.0mg/kg). Bars plot the average number of FJ-B positive cells across 12 serial sections (approximately every 6^{th} section) +/- SEM.

Figure 18: Fluoro-Jade-B positive cells in the cingulate and retrosplenial cortecies of adult female rats treated with the non-competitive NMDA receptor antagonist, memantine and co-injected with either saline or PB (1.0mg/kg). Bars plot the average number of FJ-B positive cells across 12 serial sections (approximately every 6^{th} section)

+/- SEM. **A.** FJ-B positive cells in animals exposed to a saline-saline injection (S+S), 75mg/kg memantine co-injected with saline (mem+S), or 75mg/kg memantine co-injected with PB (mem+PB). **B.** FJ-B positive cells in animals exposed to a saline-saline injection (S+S), 25mg/kg memantine co-injected with saline (mem+S), or 25mg/kg memantine co-injected with PB (mem+PB). Note that the Y-axis scales describing the mean number of FJ-B positive cells differ between **A** and **B**.

Figure 19: Effect of memantine co-injected with either saline or 1.0mg/kg PB on neuromuscular behaviors. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by observation of the following behaviors: tail elevation, tail elevation, gait, limb rotation, grip and extensor thrust.

Figure 20: Effect of memantine co-injected with either saline or 1.0mg/kg PB on sensorimotor behaviors. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by scoring the following behaviors: startle response, escape behaviors, visual placing, righting reflex and catalepsy.

Figure 21: Effect of memantine co-injected with either saline or 1.0mg/kg PB on CNS-excitability behaviors. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by scoring the presence and degree of convulsions and tremors in animals after they are removed from the home cage to the arena.

Figure 22: Effect of memantine co-injected with either saline or 1.0mg/kg PB on CNS-activity. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by scoring home cage body posture, and the presence and degree of convulsions, twitches and tremors in animals before they are removed from the home cage to the arena.

Figure 23: Effect of memantine co-injected with either saline or 1.0mg/kg PB on rearing behavior. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by keeping a tally of the total number of rears made in the first three minutes in the arena after removal from the home cage.

Figure 24: Effect of memantine co-injected with either saline or 1.0mg/kg PB on positional passivity. Rats were injected with saline; then co-injected with either saline (control), or 1.0mg/kg PB. Bars plot the average scores (+/- SEM) from raw data obtained by scoring the degree of the rats struggle when briefly suspended by the tail close to the arena cage floor.

Figure 25: The cumulative inter-event interval distribution shows a significant increase in the inter-event interval of sIPSPs from a RSC pyramidal cell after bath application of both memantine (300uM) and Pyridostigmine bromide (250uM) ($p<0.05$, K-S test).

Table 1: Functional observation battery test panel

Experiment:		Post-drug time:			
Drug 1/route:		Date:			
Drug 2/route:		Examiner init:			
Species: Rat	Animal code:	1	2	3	4
Strain: Sprague-Dawley	Body weight (g):				
Sex: female	Volume injected:				
Age: retired breeder	Time admin:				

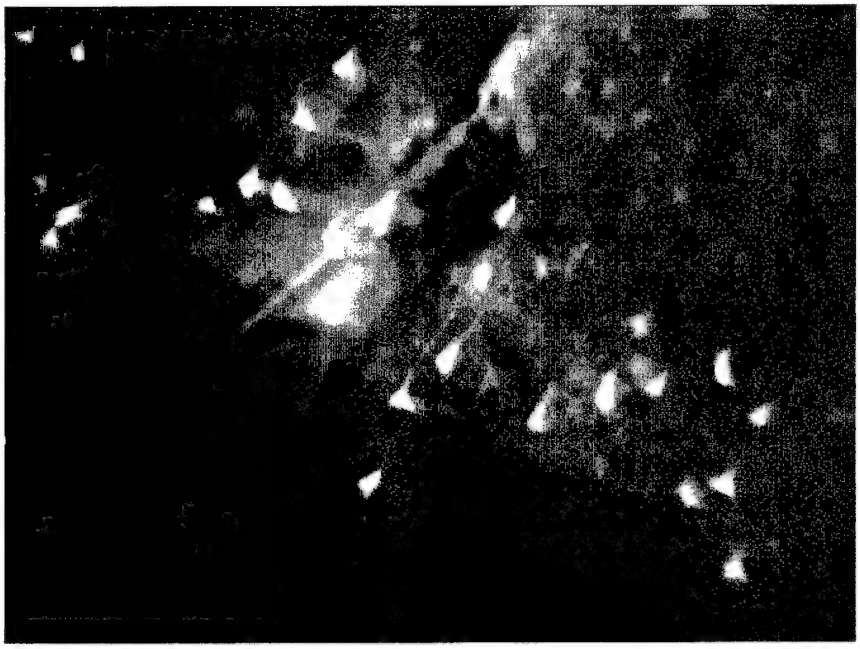
	measurement	Score				
1	Body posture	scale 1-9				
2	Bizarre behavior	present = 1, nature				
3	Tremors I	scale 1-5, nature				
4	Twitches	scale 1-5				
5	Convulsions I	clonic/tonic, scale				
6	Palpebral closure I	scale 1-4				
7	Exothalmos	present = 1				
8	Eye crustiness	present = 1				
9	Arousal	scale 1-6				
10	Convulsions II	clonic/tonic, scale				
11	Tremors II	scale 1-5, nature				
12	Time to movement	seconds				
13	Palpebral closure I	scale 1-4				
14	Piloerection	present = 1				
15	Fur appearance	scale 1-3				
16	Rearing/3 min.	number/3 min				
17	Grooming/3 min.	number/3 min				
18	Tail elevation	scale 1-5				
19	Startle response	scale 1-3				
20	Provoked biting	scale 1-5				
21	Finger approach	scale 1-5				
22	Finger withdrawal	scale 1-5				
23	Touch-escape	scale 1-7				
24	Gait	scale 1-6				

	measurement	Animal code:	1	2	3	4
25	Total gait incapacit	scale 1-4				
26	Limb rotation	scale 1-5, code				
27	Positional passivity	scale 1-5				
28	Visual placing	scale 1-5				
29	Grip strength	scale 1-5				
30	Body tone	scale 1-5				
31	Hypothermia	present = 1				
32	Lacrimation	present = 1				
33	Toe-pinch	scale 1-5				
34	Pencil Grip	scale 1-3				
35	Extensor thrust	scale 1-4				
36	Diarrhea	present = 1				
37	Salivation	scale 1-5				
38	Tail pinch	scale 1-7				
39	Righting reflex	scale 1-4				
40	Catalepsy	scale 1-4				
41	Grasp irritability	scale 1-4				
42	Provoked freezing	scale 1-4				
43	Vocalizations	number of/3 min.				
44	Urination/defecat.	number of/3 min.				
45	Death	present +				

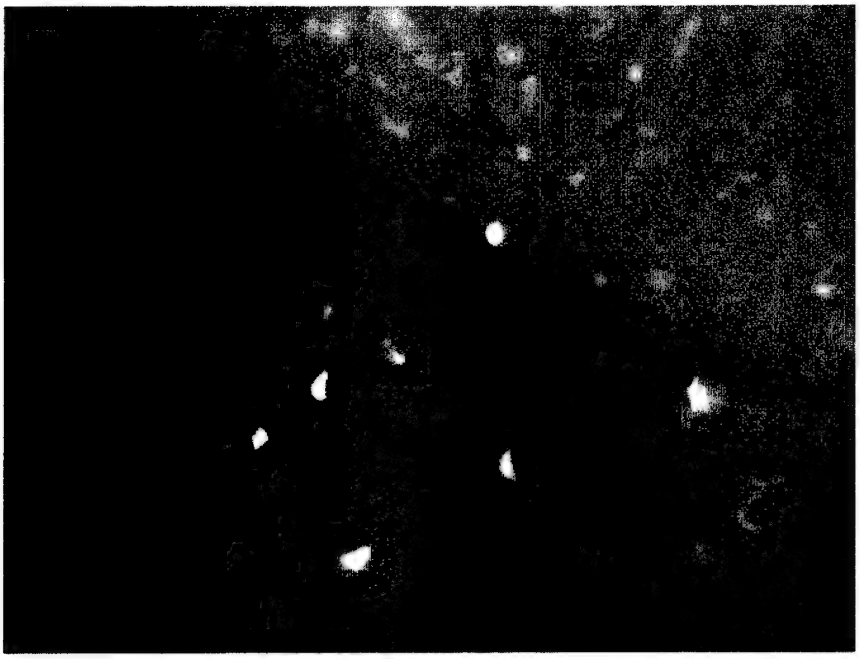
Table 2: Functional behavioral domains

Functional domain	Behavior
Neuromuscular	Tail elevation
	Gait score
	Gait incapacity
	Limb rotation
	Grip strength
	Body tone
	Pencil Grip
	Extensor thrust
Sensorimotor	Startle response
	Finger approach
	Finger withdrawal
	Touch-escape
	Visual placing
	Righting reflex
	Catalepsy
Pain	Toe-pinch
	Tail-pinch
CNS excitability	Convulsions II
	TremorsII
CNS activity	Body posture
	TremorsI
	Twitches
	Convulsions I
	Rearing

Functional domain	Behavior
Autonomic/general	Palpebral closure I
	Exothalmos
	Eye crustiness
	Piloerection
	Hypothermia
	Lacrimation
Autonomic/G.I.	Diarrhea
	Salivation
	Defecation
Behavioral:	Bizarre behavior
	Palpebral closure II
	Fur appearance
	Grooming
	Provoked biting
	Positional passivity
Motor affective:	Arousal
	Time to movement
	Grasp irritability
	Provoked freezing
	Vocalizations



A



B

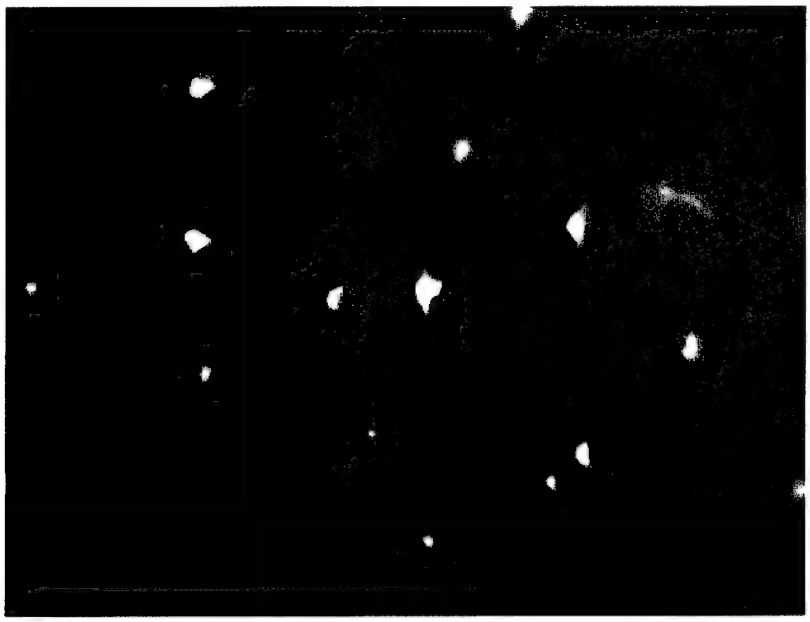
C

A

B

Figure 2

A



B

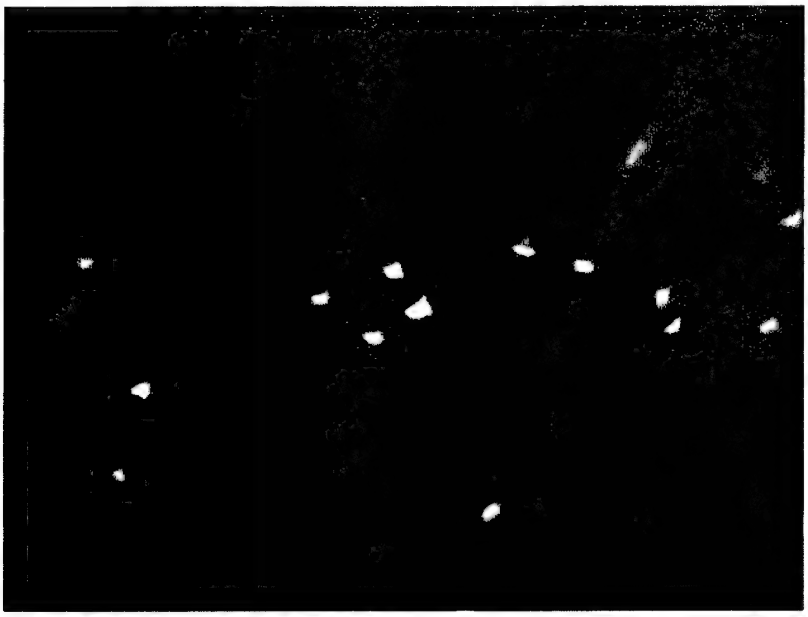
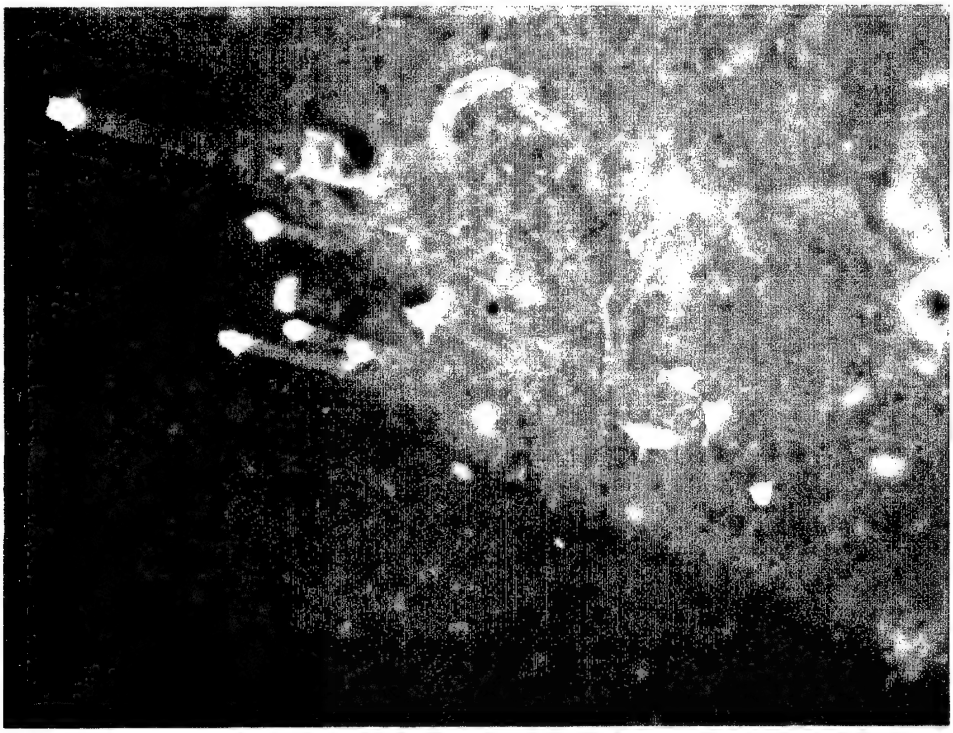


Figure 3

A



B

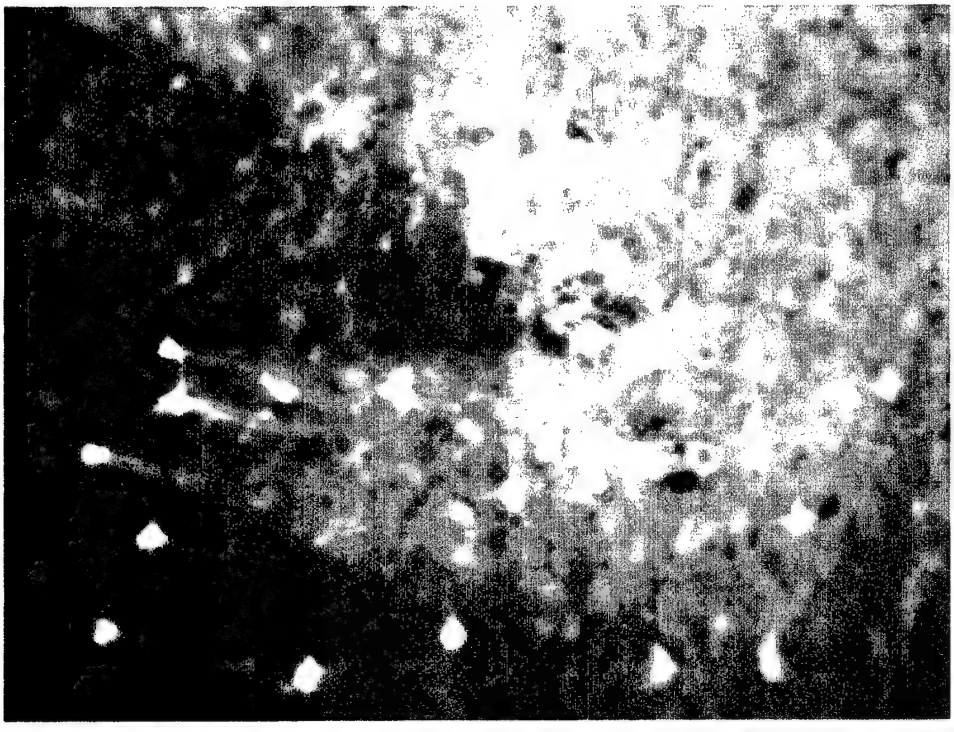
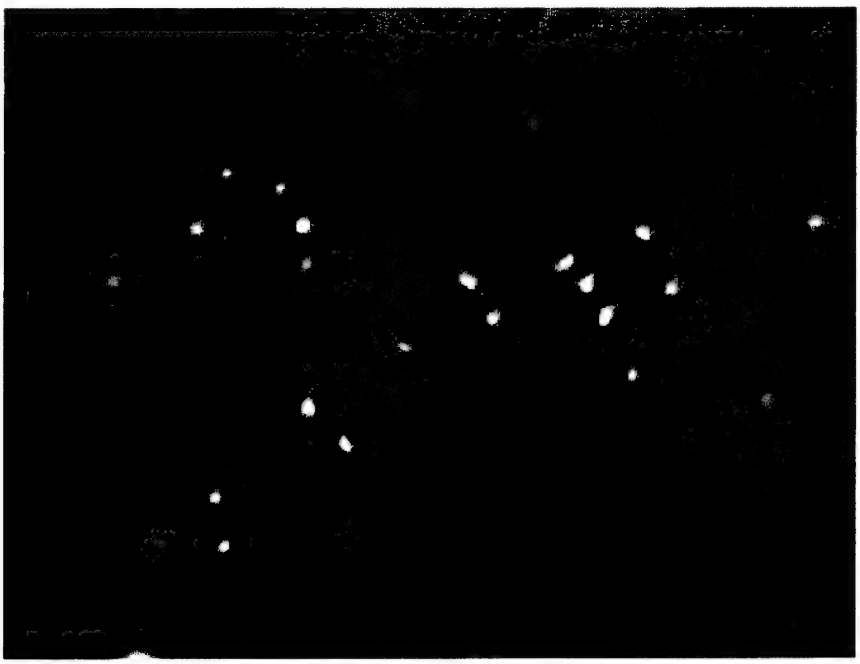


Figure 4

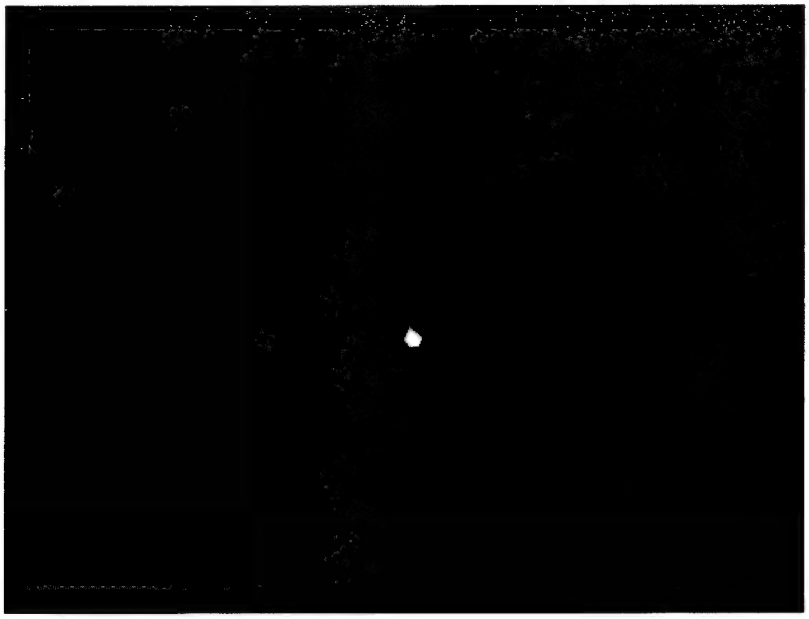
A



B

Figure 5

A



B

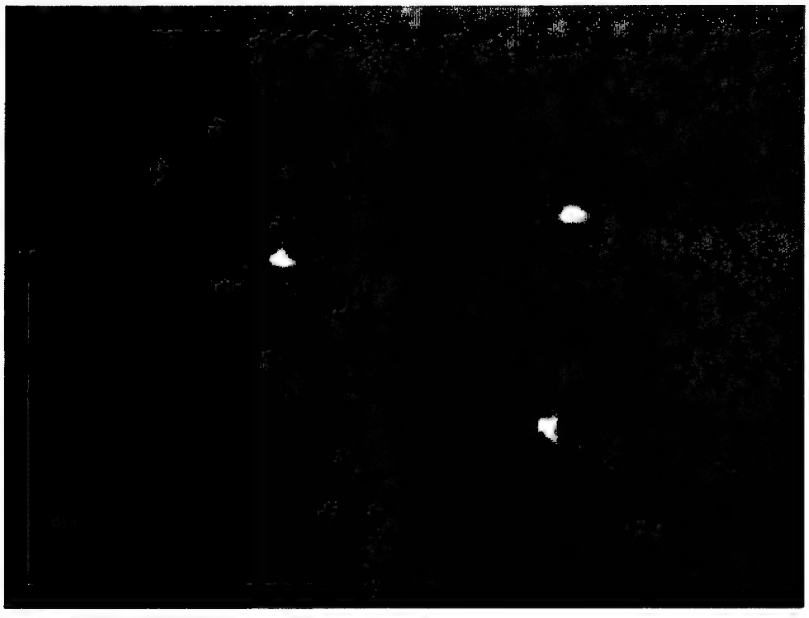


Figure 6

Figure 7

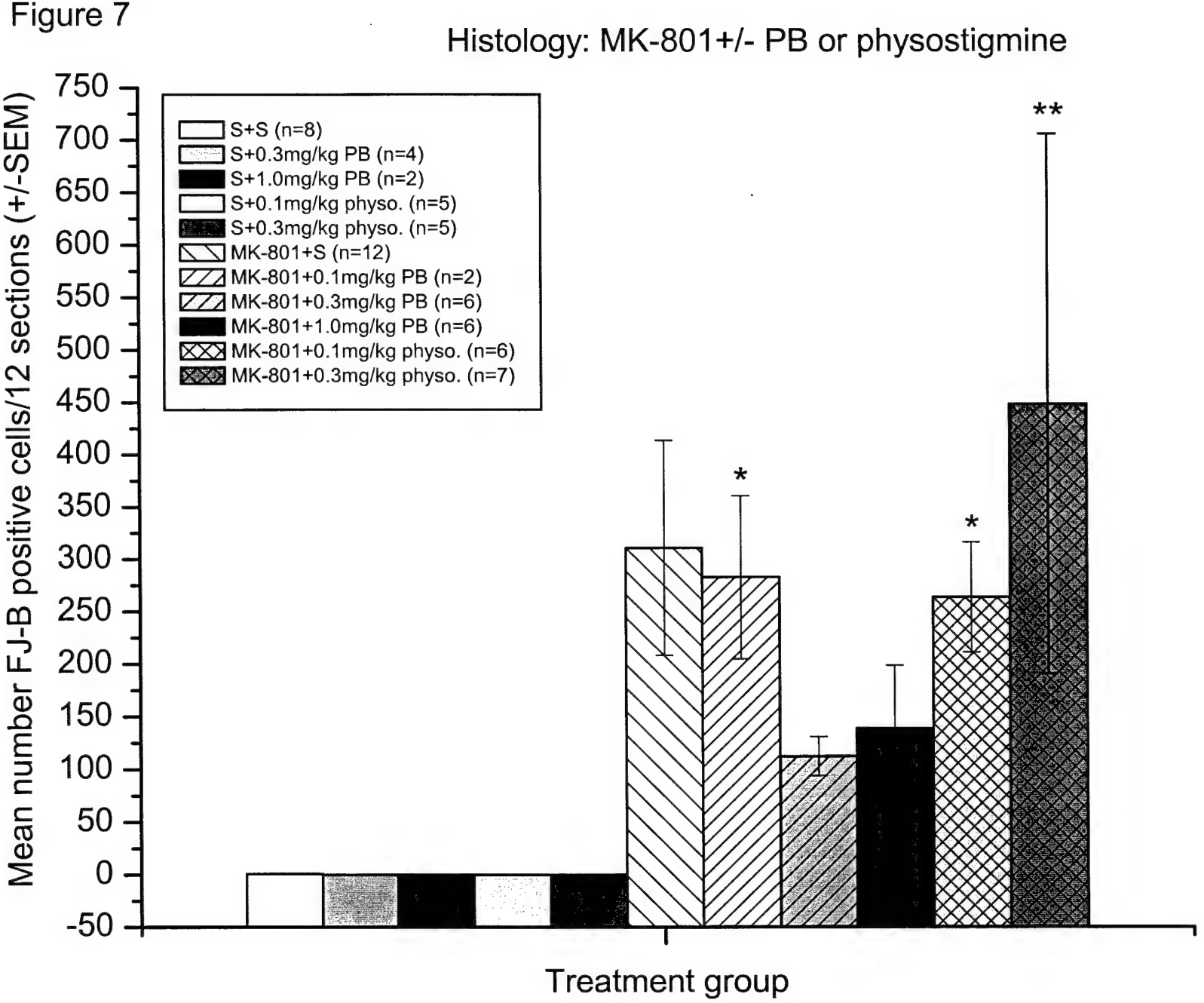

Histology: MK-801+/- PB or physostigmine
Mean number FJ-B positive cells/12 sections (+/-SEM)
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
-50
S+S (n=8)
S+0.3mg/kg PB (n=4)
S+1.0mg/kg PB (n=2)
S+0.1mg/kg physo. (n=5)
S+0.3mg/kg physo. (n=5)
MK-801+S (n=12)
MK-801+0.1mg/kg PB (n=2)
MK-801+0.3mg/kg PB (n=6)
MK-801+1.0mg/kg PB (n=6)
MK-801+0.1mg/kg physo. (n=6)
MK-801+0.3mg/kg physo. (n=7)
*
*
**
Treatment group

Figure 8

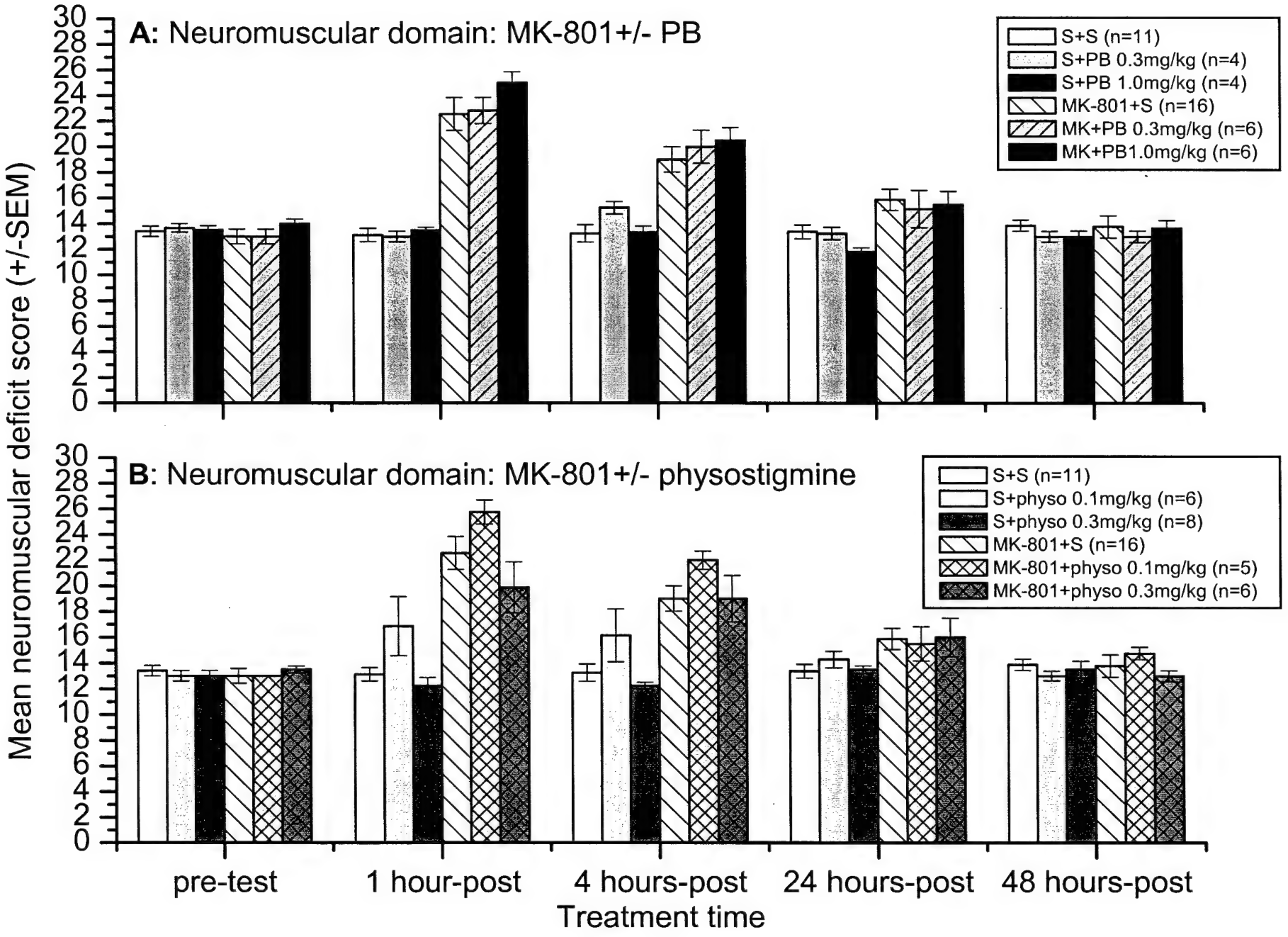

A: Neuromuscular domain: MK-801+/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB 1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB1.0mg/kg (n=6)
B: Neuromuscular domain: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK-801+physo 0.1mg/kg (n=5)
MK-801+physo 0.3mg/kg (n=6)
Mean neuromuscular deficit score (+/-SEM)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 9

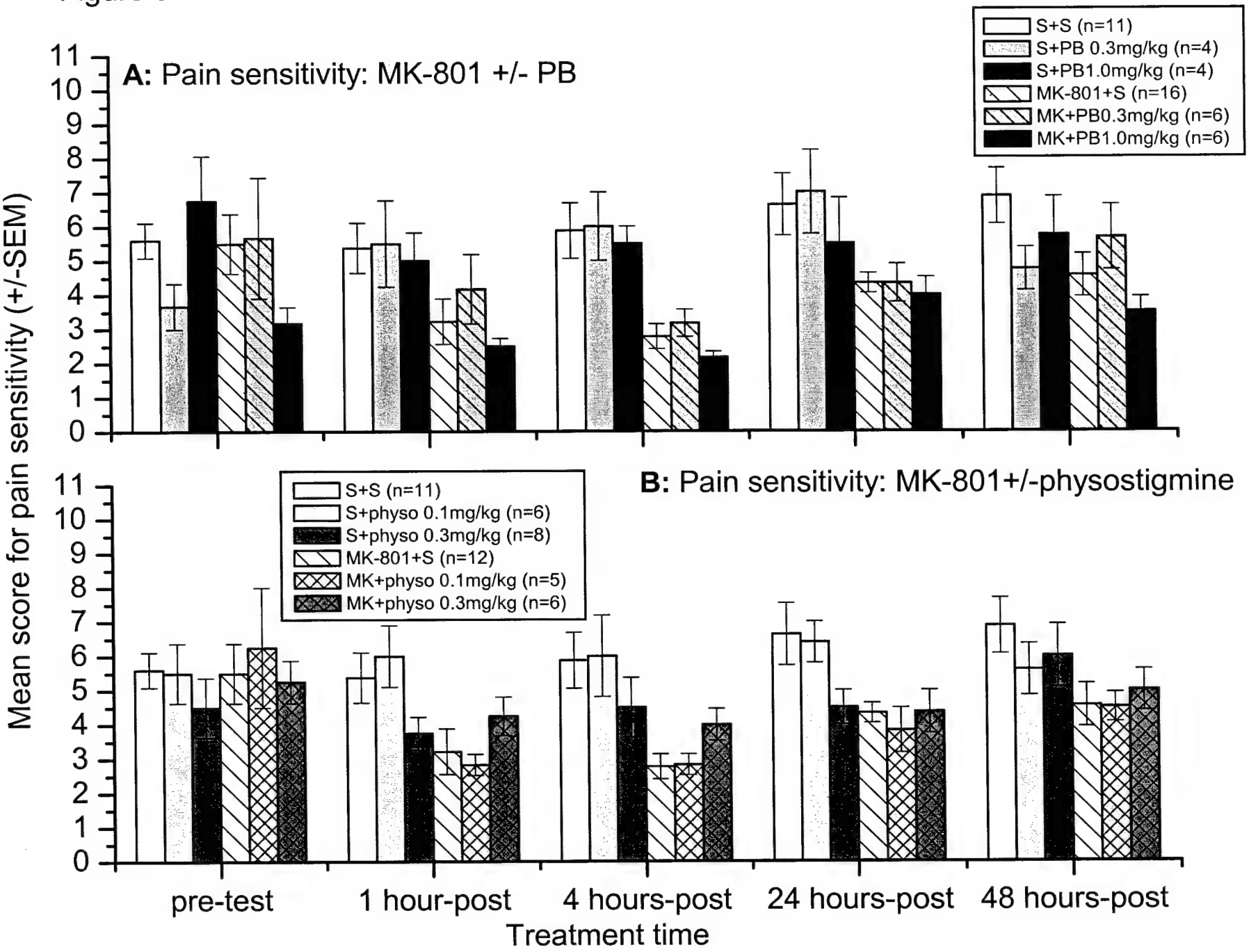

A: Pain sensitivity: MK-801 +/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB0.3mg/kg (n=6)
MK+PB1.0mg/kg (n=6)
B: Pain sensitivity: MK-801+/-physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=12)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Mean score for pain sensitivity (+/-SEM)
0
1
2
3
4
5
6
7
8
9
10
11
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 10

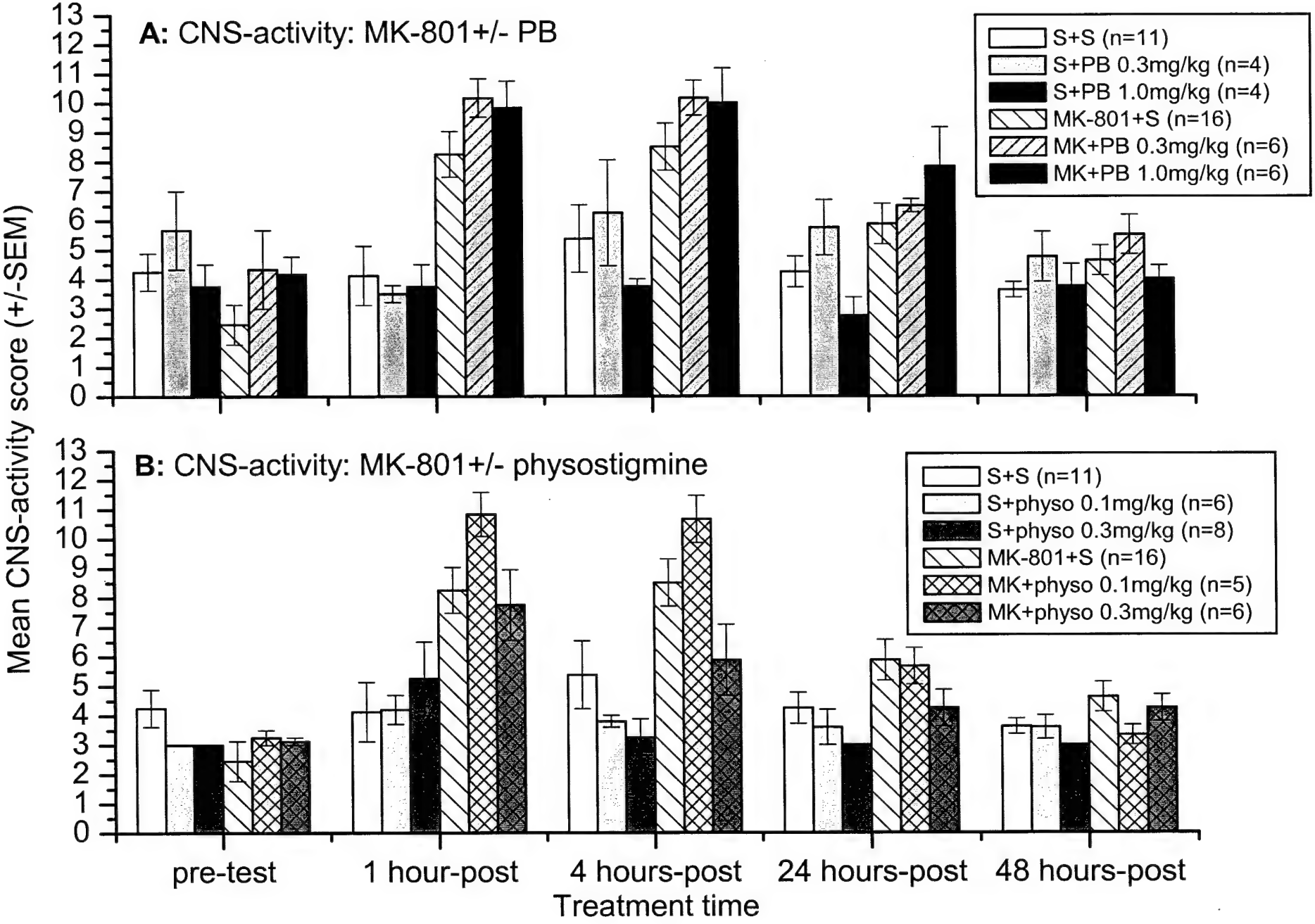

A: CNS-activity: MK-801+/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB 1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB 1.0mg/kg (n=6)
B: CNS-activity: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Mean CNS-activity score (+/-SEM)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 11

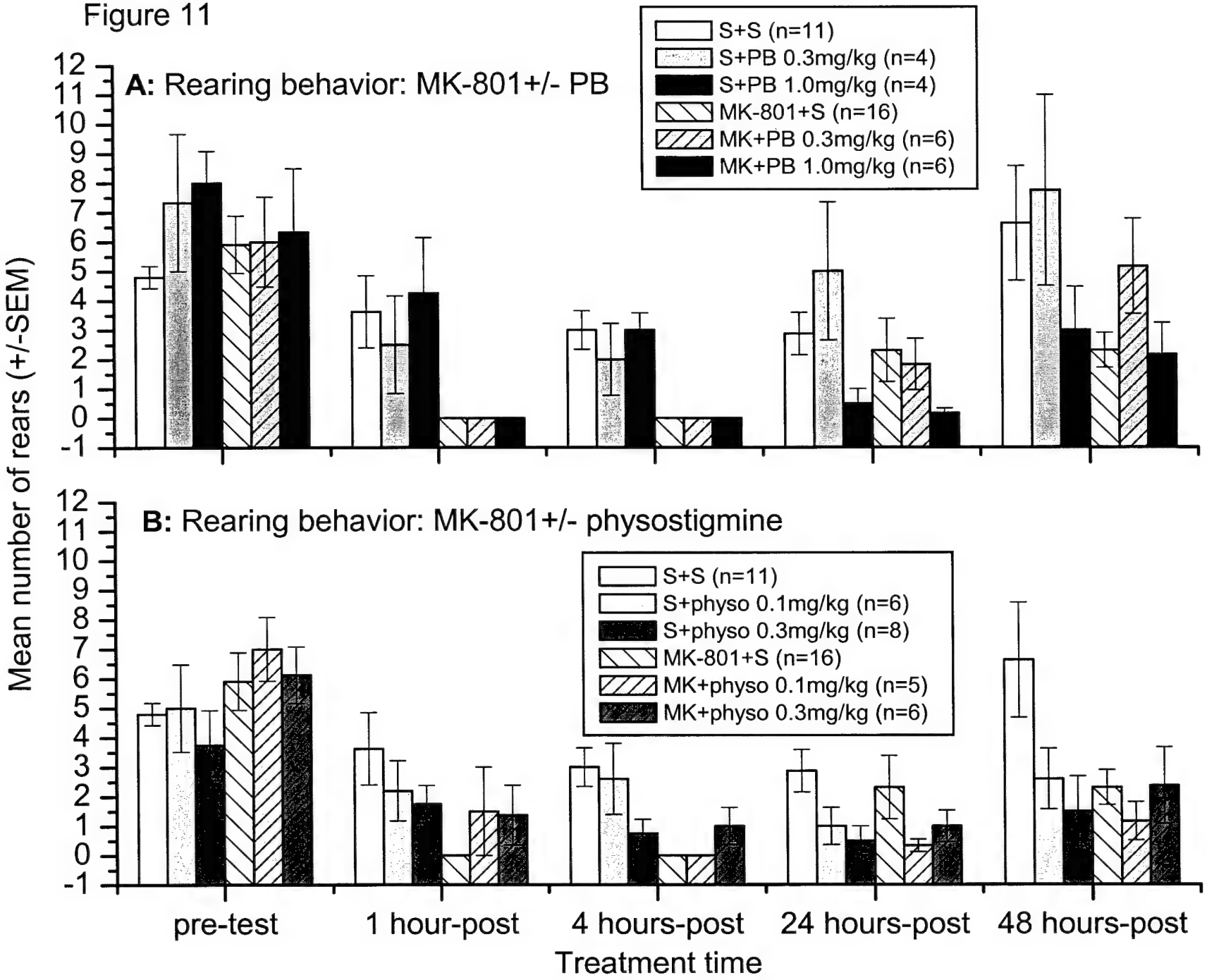

A: Rearing behavior: MK-801+/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB 1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB 1.0mg/kg (n=6)
B: Rearing behavior: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Mean number of rears (+/-SEM)
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 12

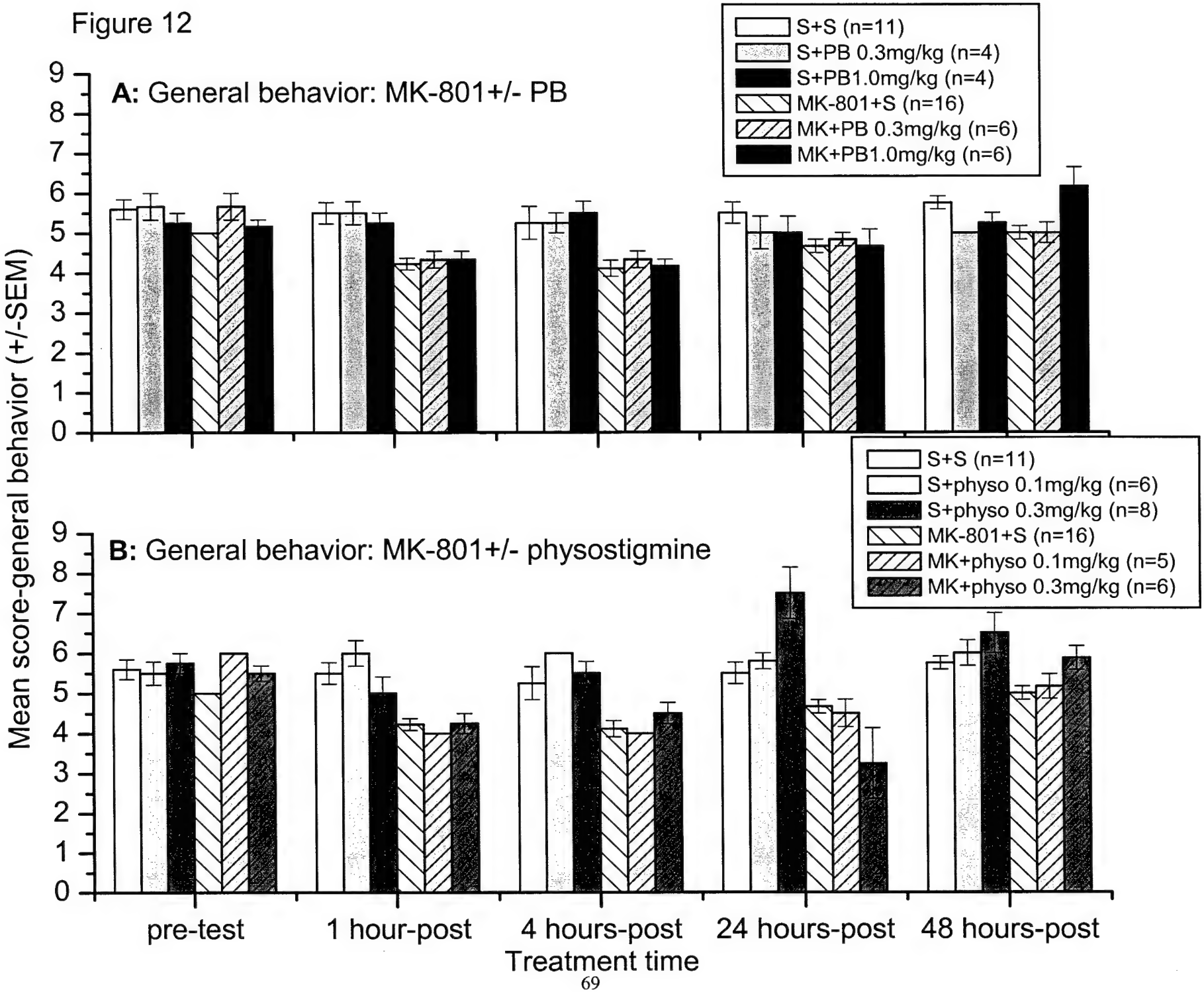

A: General behavior: MK-801+/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB1.0mg/kg (n=6)
B: General behavior: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Mean score-general behavior (+/-SEM)
0
1
2
3
4
5
6
7
8
9
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 13

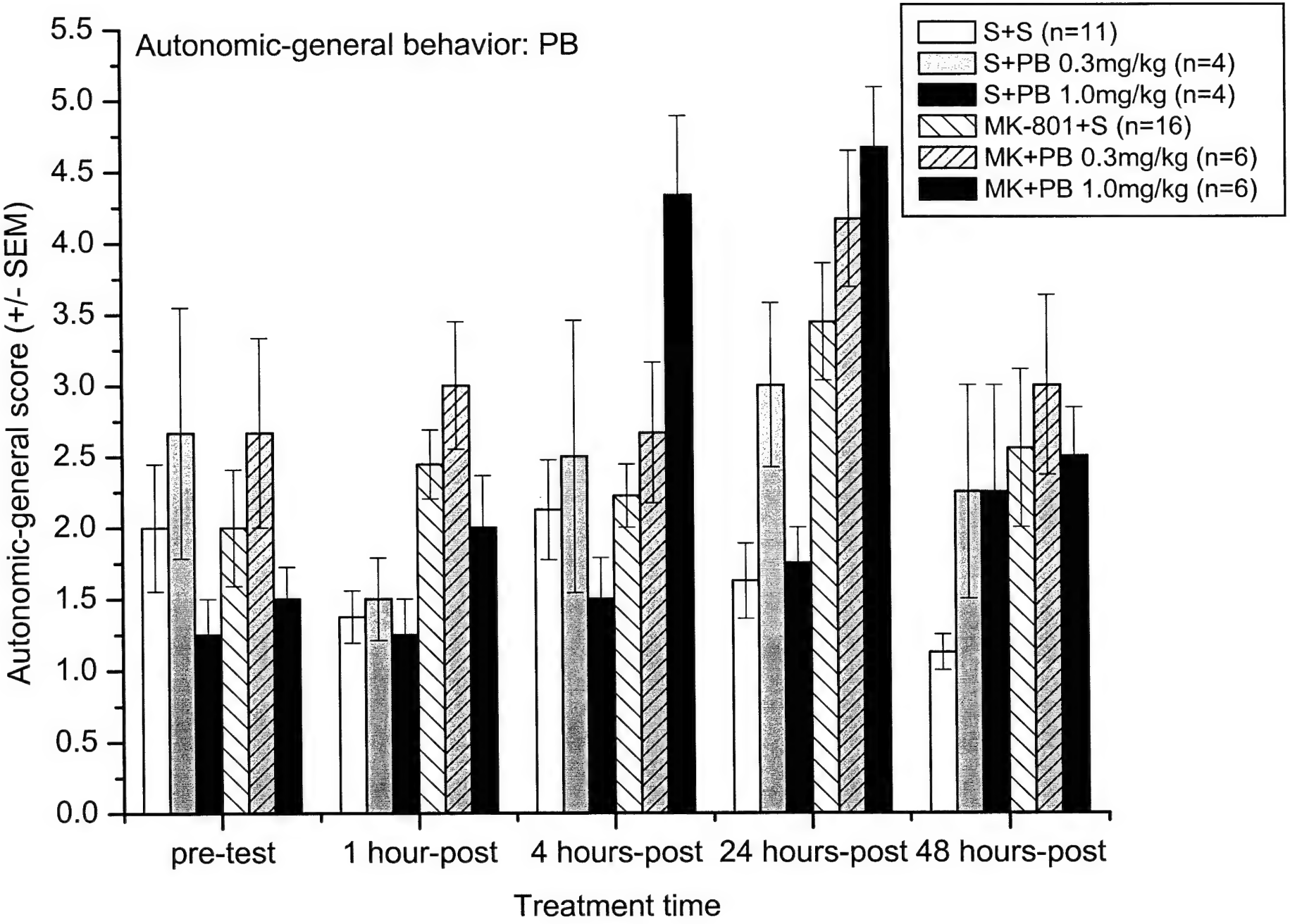

Autonomic-general behavior: PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB 1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB 1.0mg/kg (n=6)
Autonomic-general score (+/- SEM)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 14

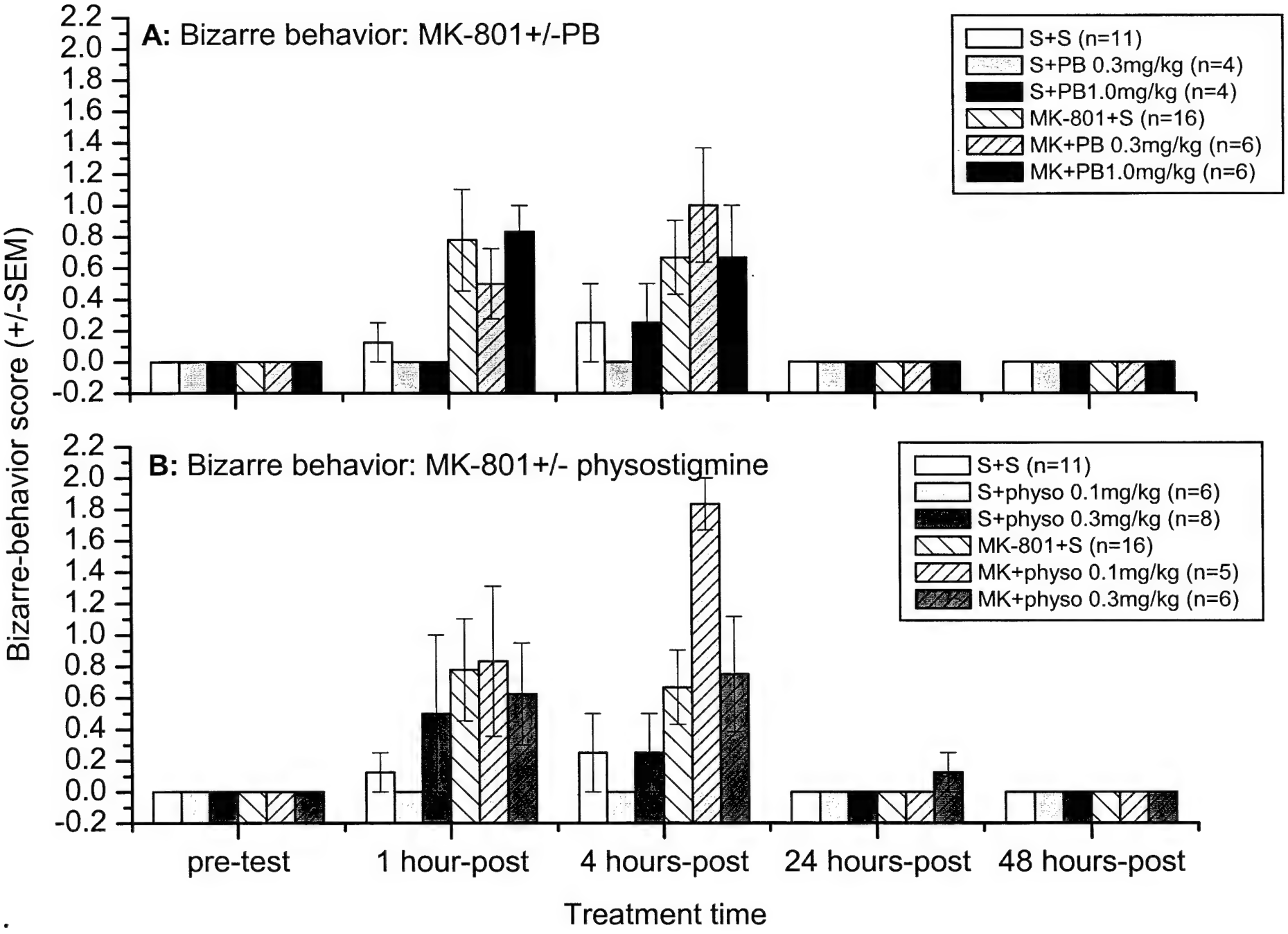

A: Bizarre behavior: MK-801+/-PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB1.0mg/kg (n=6)
B: Bizarre behavior: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Bizarre-behavior score (+/-SEM)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 15

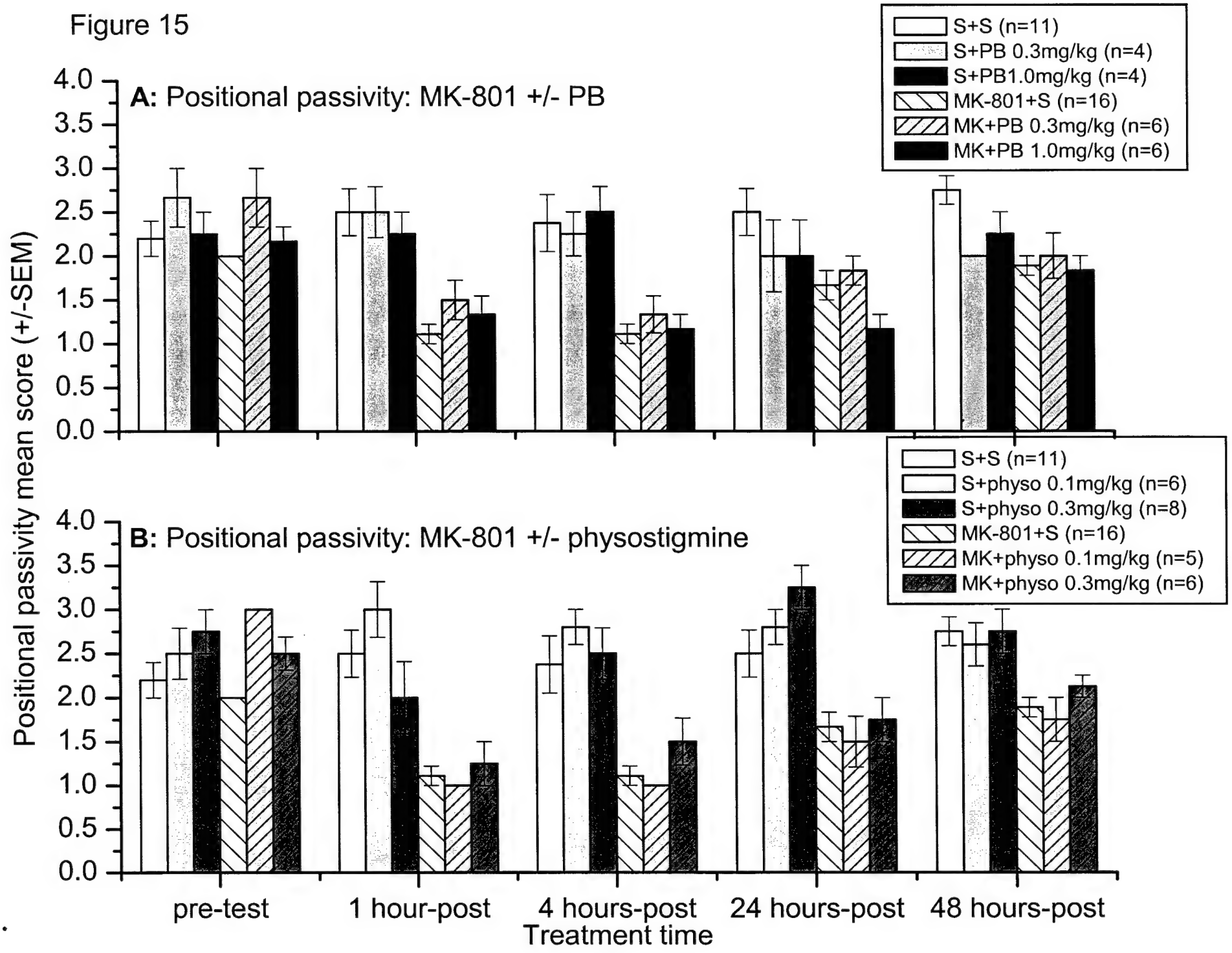

A: Positional passivity: MK-801 +/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB 1.0mg/kg (n=6)
B: Positional passivity: MK-801 +/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Positional passivity mean score (+/-SEM)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 16

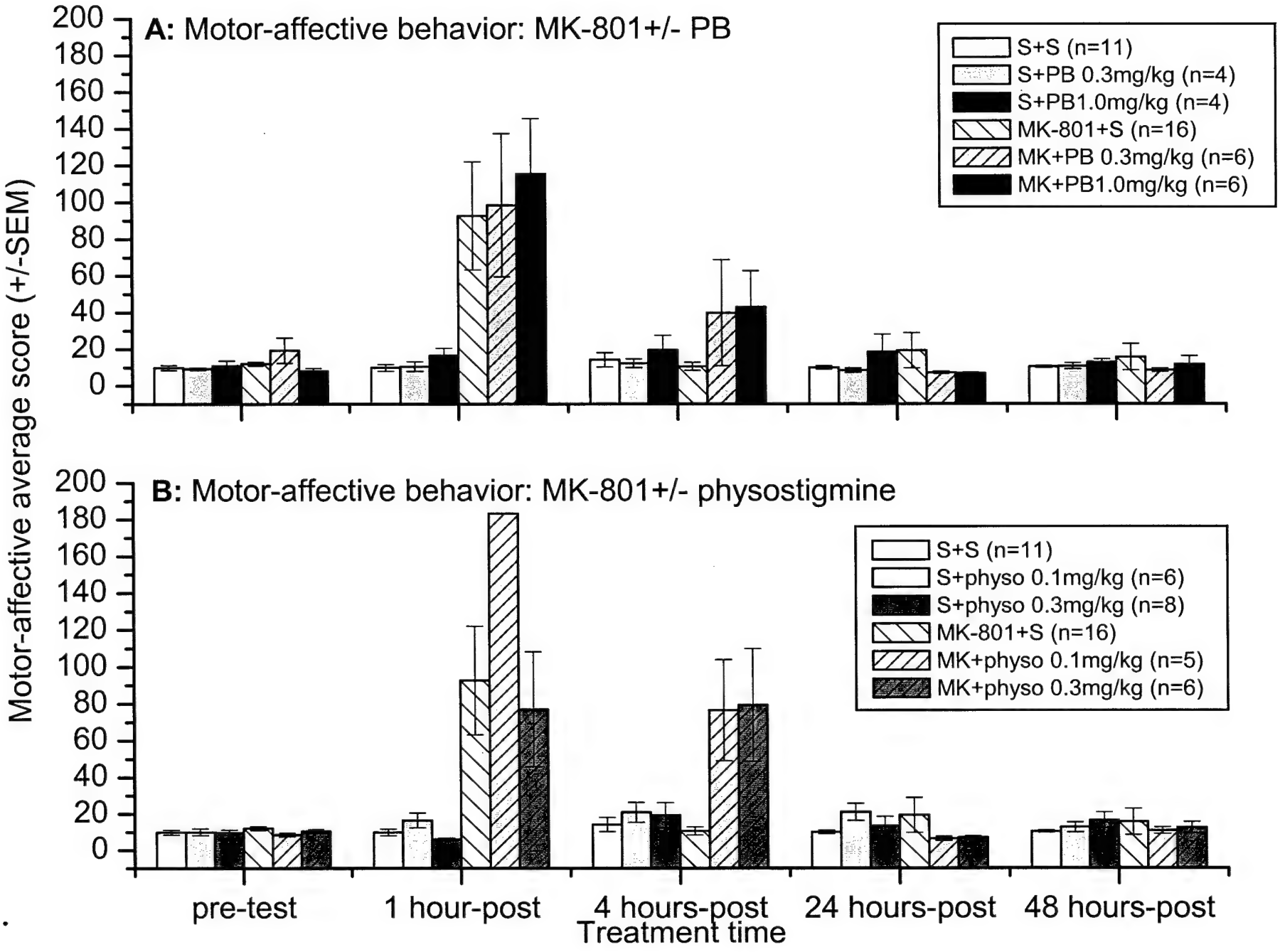

A: Motor-affective behavior: MK-801+/- PB
S+S (n=11)
S+PB 0.3mg/kg (n=4)
S+PB1.0mg/kg (n=4)
MK-801+S (n=16)
MK+PB 0.3mg/kg (n=6)
MK+PB1.0mg/kg (n=6)
B: Motor-affective behavior: MK-801+/- physostigmine
S+S (n=11)
S+physo 0.1mg/kg (n=6)
S+physo 0.3mg/kg (n=8)
MK-801+S (n=16)
MK+physo 0.1mg/kg (n=5)
MK+physo 0.3mg/kg (n=6)
Motor-affective average score (+/-SEM)
200
180
160
140
120
100
80
60
40
20
0
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 17

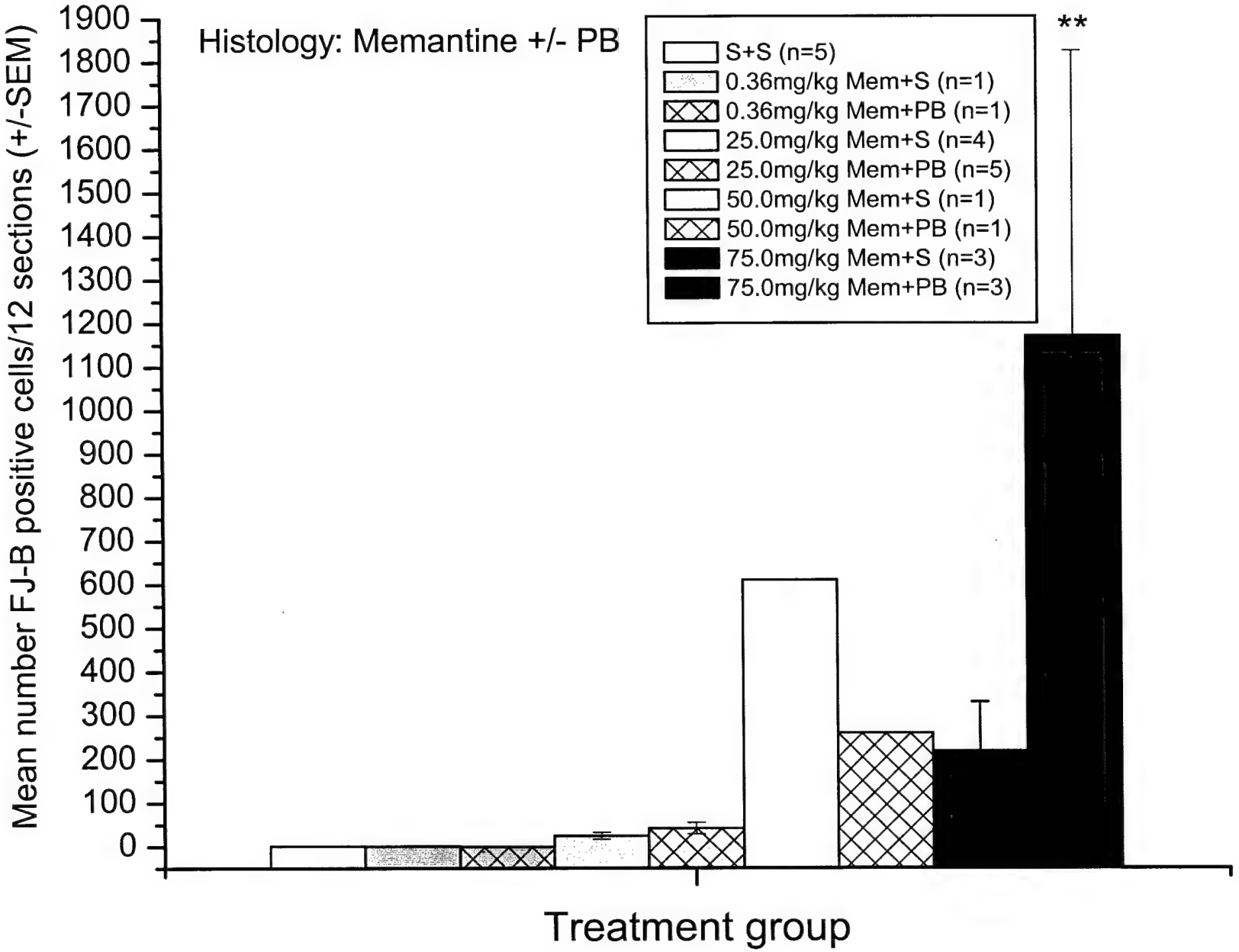

Histology: Memantine +/- PB
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25.0mg/kg Mem+S (n=4)
25.0mg/kg Mem+PB (n=5)
50.0mg/kg Mem+S (n=1)
50.0mg/kg Mem+PB (n=1)
75.0mg/kg Mem+S (n=3)
75.0mg/kg Mem+PB (n=3)
**
Mean number FJ-B positive cells/12 sections (+/-SEM)
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Treatment group

Figure 18

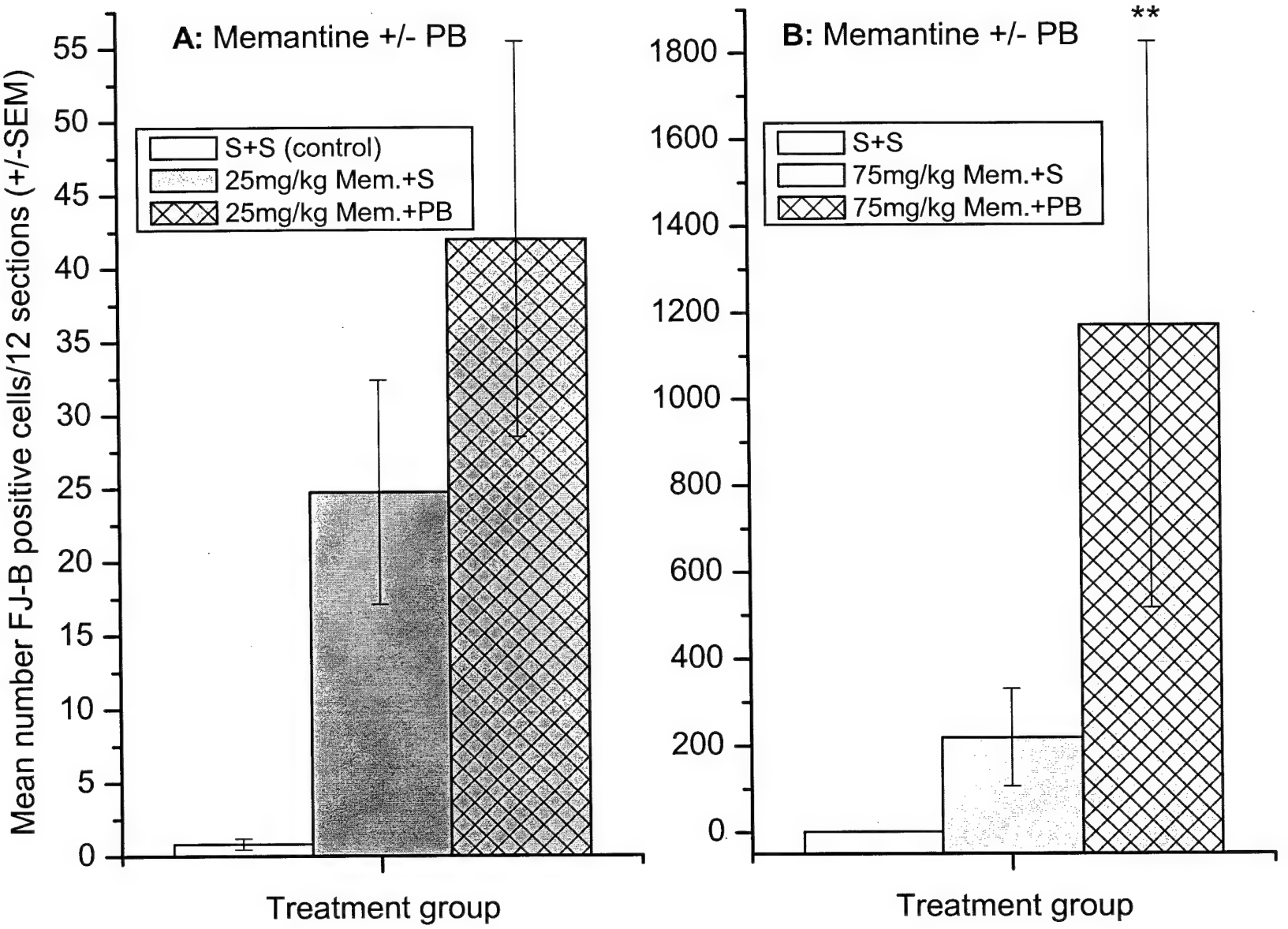

A: Memantine +/- PB
S+S (control)
25mg/kg Mem.+S
25mg/kg Mem.+PB
Mean number FJ-B positive cells/12 sections (+/-SEM)
0
5
10
15
20
25
30
35
40
45
50
55
Treatment group
B: Memantine +/- PB
**
S+S
75mg/kg Mem.+S
75mg/kg Mem.+PB
0
200
400
600
800
1000
1200
1400
1600
1800
Treatment group

Figure 19

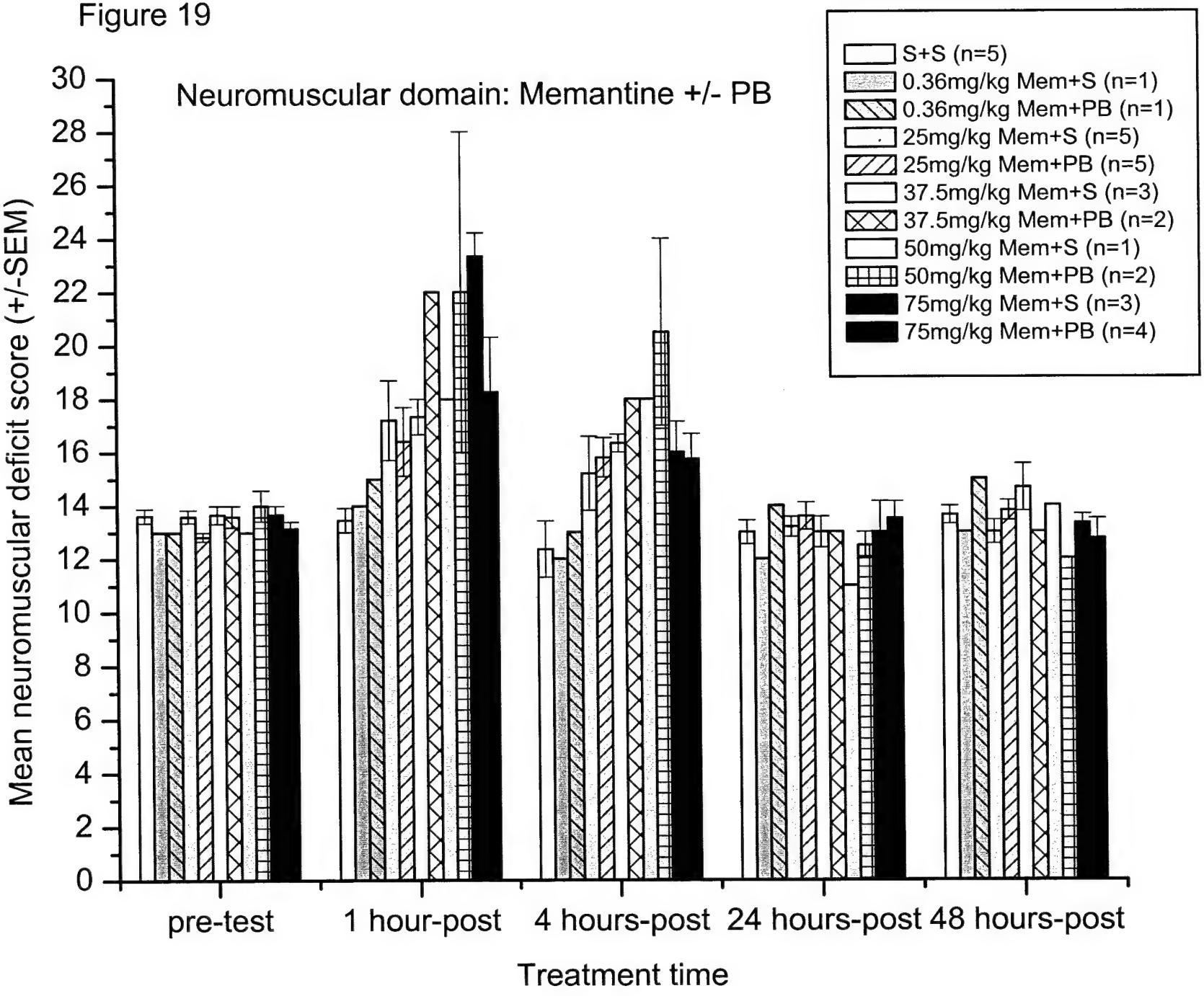

Neuromuscular domain: Memantine +/- PB
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)
Mean neuromuscular deficit score (+/-SEM)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 20

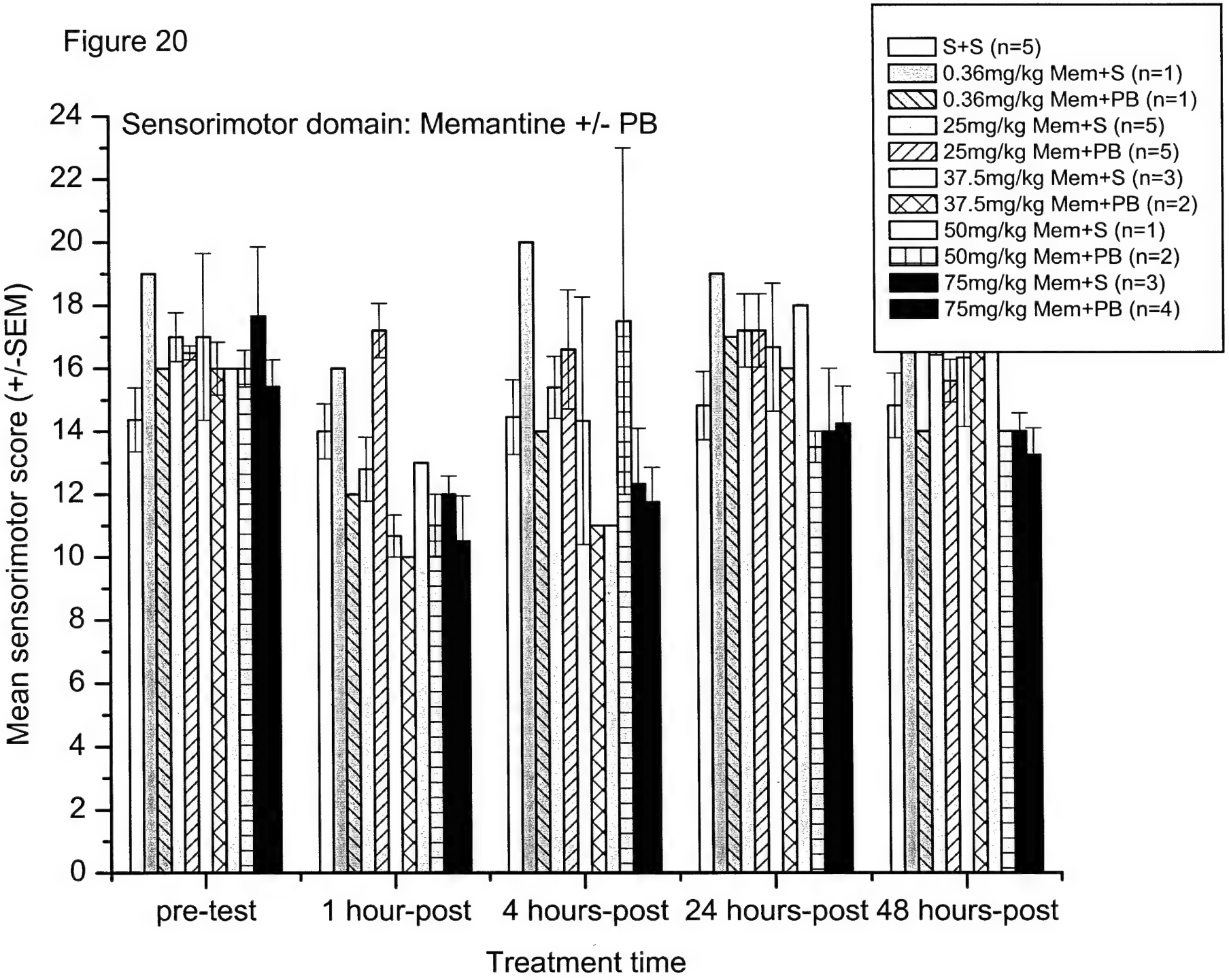

Sensorimotor domain: Memantine +/- PB
Mean sensorimotor score (+/-SEM)
0
2
4
6
8
10
12
14
16
18
20
22
24
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)

Figure 21

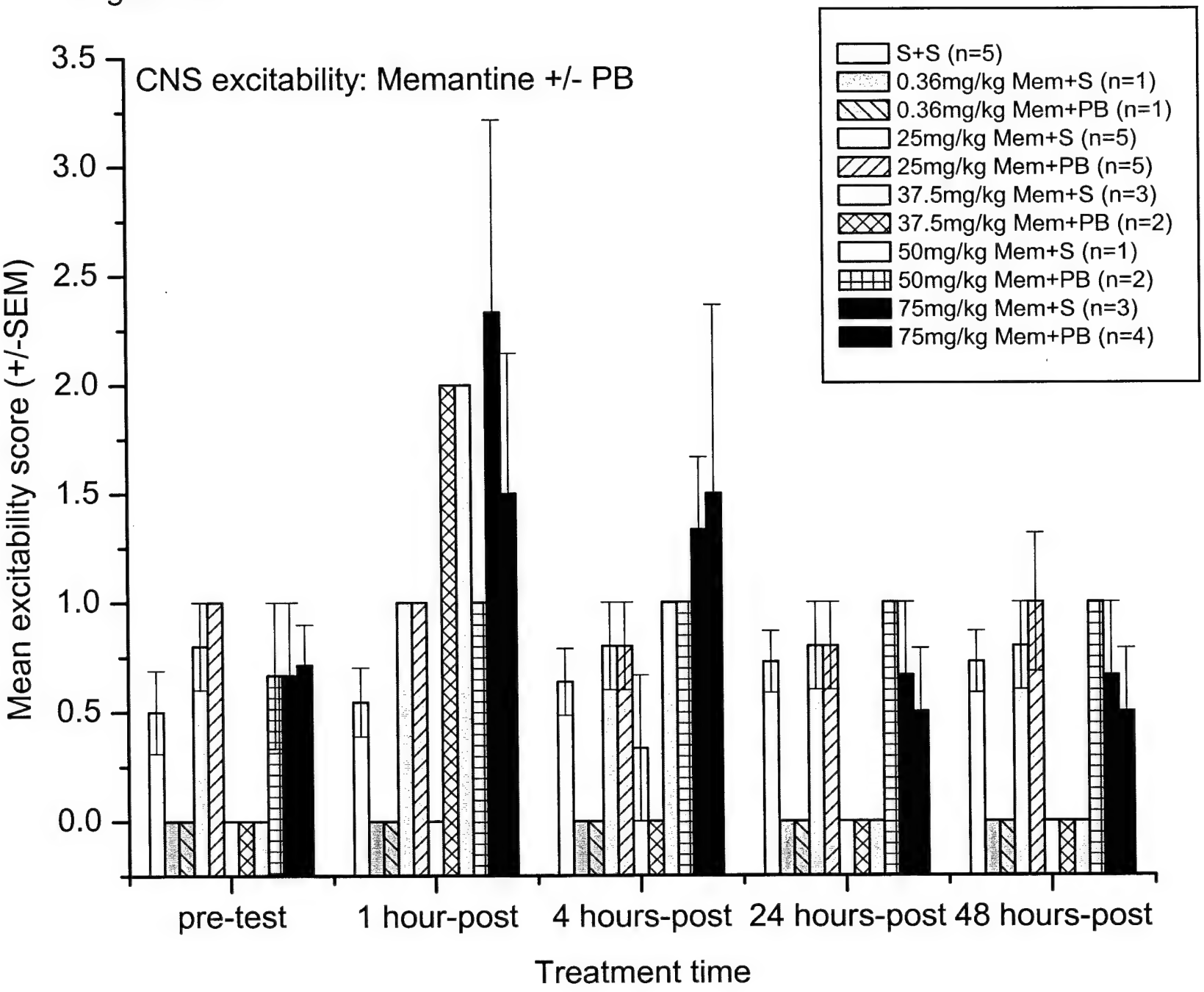

CNS excitability: Memantine +/- PB
Mean excitability score (+/-SEM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)

Figure 22

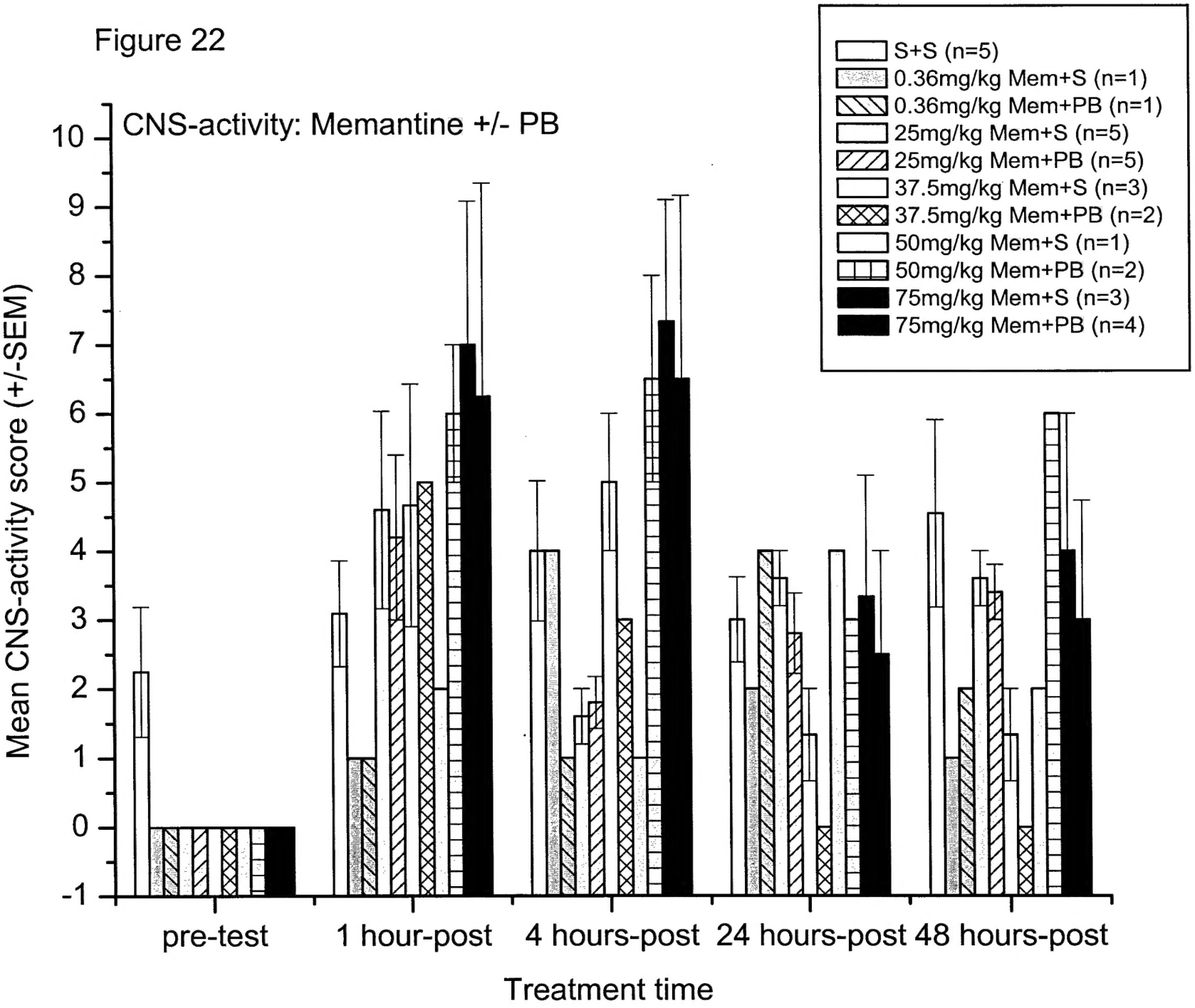

CNS-activity: Memantine +/- PB
Mean CNS-activity score (+/-SEM)
10
9
8
7
6
5
4
3
2
1
0
-1
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)

Figure 23

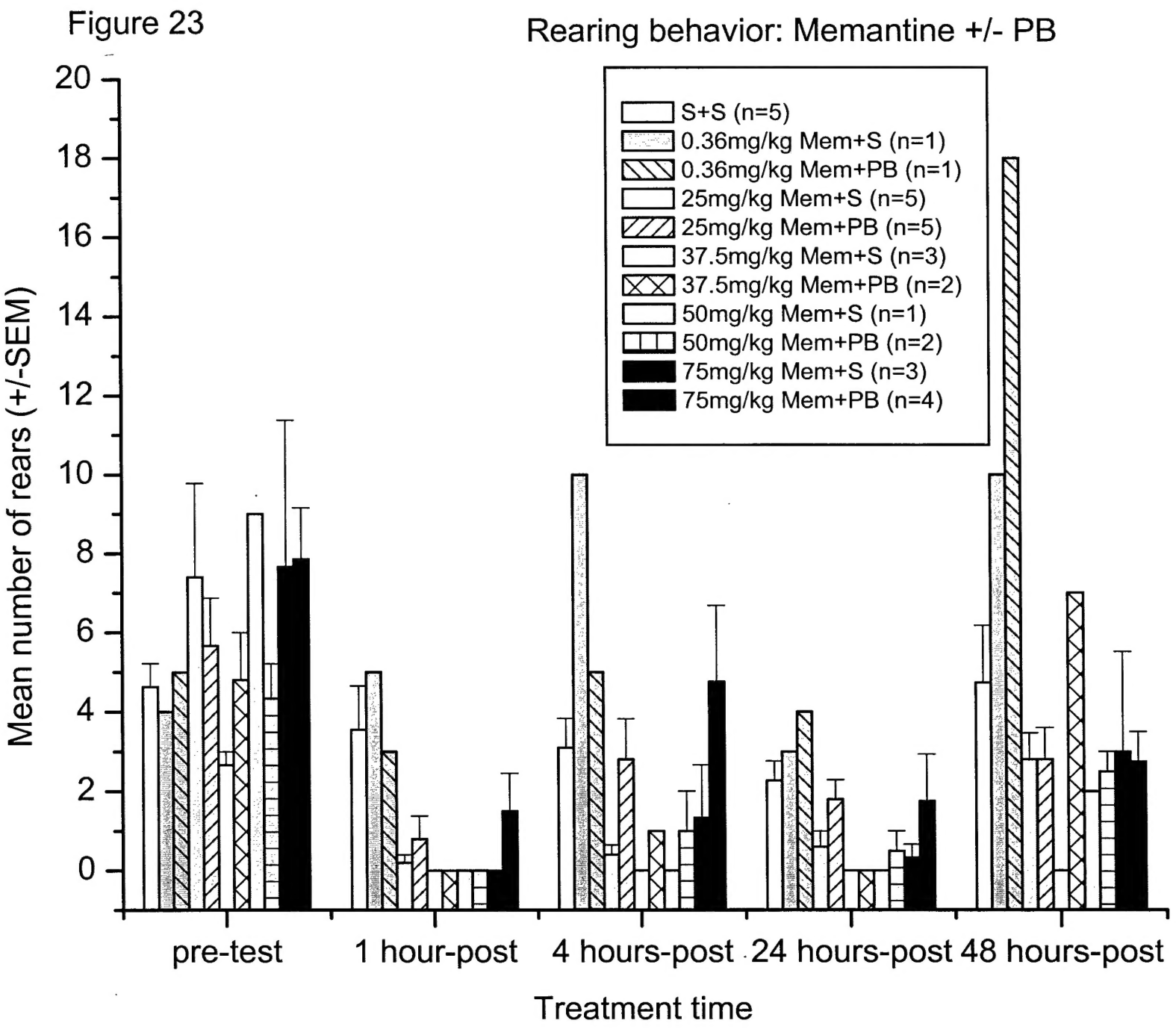

Rearing behavior: Memantine +/- PB
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)
Mean number of rears (+/-SEM)
0
2
4
6
8
10
12
14
16
18
20
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 24

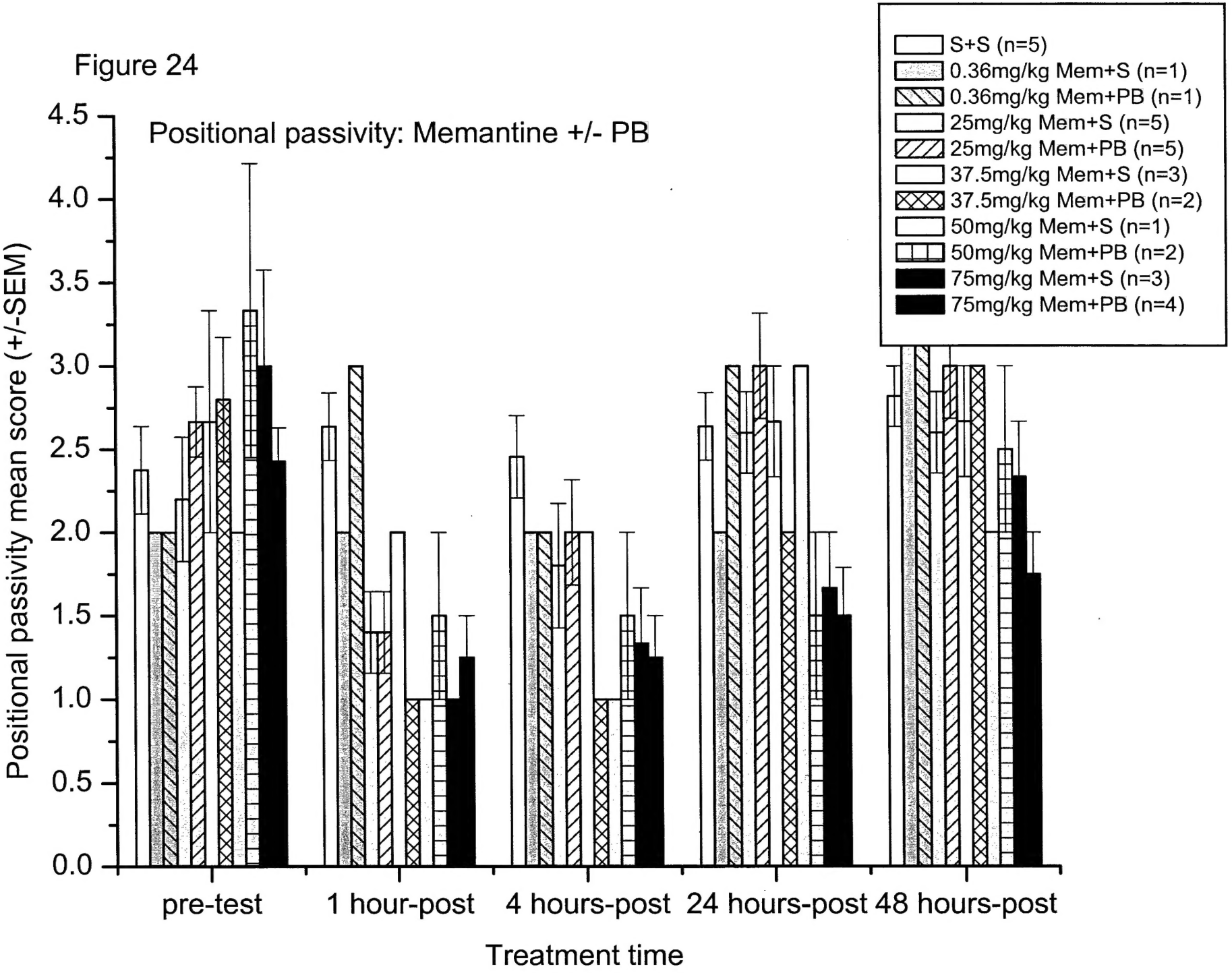
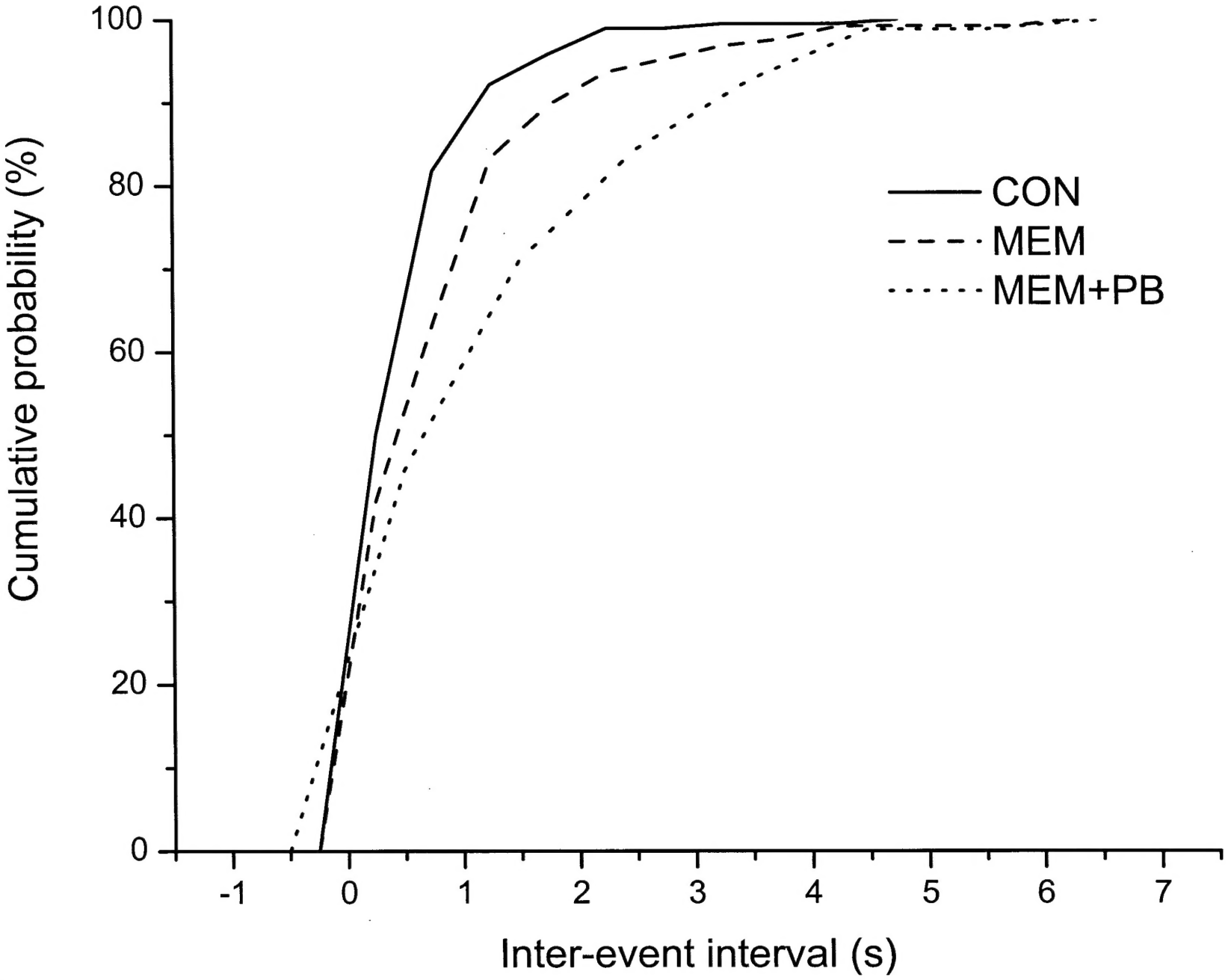

Positional passivity: Memantine +/- PB
S+S (n=5)
0.36mg/kg Mem+S (n=1)
0.36mg/kg Mem+PB (n=1)
25mg/kg Mem+S (n=5)
25mg/kg Mem+PB (n=5)
37.5mg/kg Mem+S (n=3)
37.5mg/kg Mem+PB (n=2)
50mg/kg Mem+S (n=1)
50mg/kg Mem+PB (n=2)
75mg/kg Mem+S (n=3)
75mg/kg Mem+PB (n=4)
Positional passivity mean score (+/-SEM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pre-test
1 hour-post
4 hours-post
24 hours-post
48 hours-post
Treatment time

Figure 25


100
80
60
40
20
0
Cumulative probability (%)
-1
0
1
2
3
4
5
6
7
Inter-event interval (s)
CON
MEM
MEM+PB